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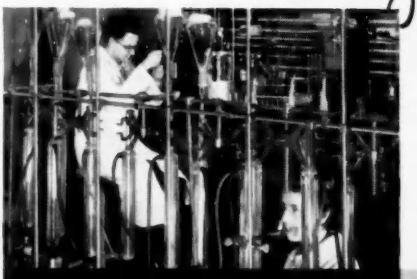

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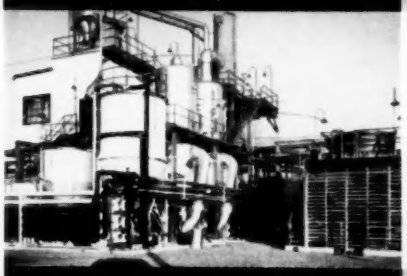

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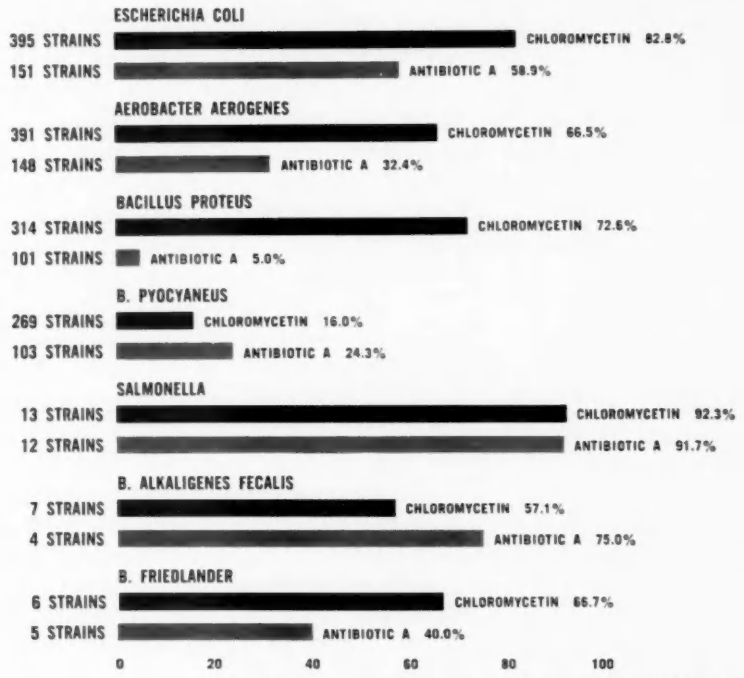

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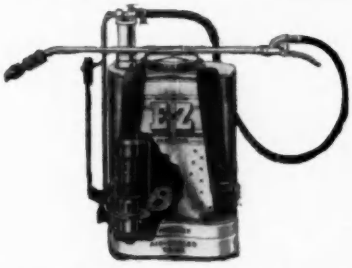

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JAPANESE B AND MUMPS ENCEPHALITIS

A Clinicopathological Report of Simultaneous Outbreaks on the Island of Guam[1, 2]

DONALD C. EDGREN, VINCENT S. PALLADINO, AND ARTHUR ARNOLD

Before World War II, Guam was a fairly isolated island of approximately 25,000 natives and a few military personnel. Consequently the incoming traffic could be closely watched for any acute contagious diseases. Since the war the increasing influx of people has resulted in an increase in the number of contagious diseases. From November, 1947 until March, 1949 there were three epidemics (measles, mumps, and chicken pox); in previous years such outbreaks were a rarity. This report was written to emphasize the fact that "localized" virus infections (such as Japanese B encephalitis) may spread to distant parts of the world. The epidemic also gave us a unique opportunity to compare the clinical aspects of two, separate, central-nervous-system virus infections occurring simultaneously.

Throughout 1947 occasional sporadic cases of mumps were reported. In November the number of cases increased and reached epidemic proportions within 1 month. Before the end of this outbreak in April, 1948 a total of 1,500 cases were reported and it is probable that at least double this number of persons were infected. Concurrent with the mumps outbreak there was an epidemic of encephalitis concerning which this report is written.

The first encephalitis case was admitted to Guam Memorial Hospital on December 6, 1947 and was recognized as such on December 10, 1947. The next case was admitted 12 days later and by December 26, 1947 eight cases were admitted to Guam Memorial Hospital. Four of these had had mumps about a week before admission; two others had had close family contact. It was thought that these were cases of mumps encephalitis, but the extreme severity and high incidence of this disease cast some doubt on this presumption. Further steps were taken to study these cases and determine their exact etiology. On December 30, 1947, sera for virus studies were collected from the patients and plans were made to draw additional specimens after 2 and 4 weeks from these cases as well as from all future cases.

The number of cases continued to increase and by January 15, 1948 there had been 28 cases with three deaths at Guam Memorial Hospital and two cases at the U. S. Naval Hospital. Further blood specimens were drawn on January 16, 1948 and on January 19, 1948. These bloods and the frozen glycerinated brain and spinal cords were flown to Dr. W. McD. Hammon, of the Hooper Foundation in San Francisco. Dr. Hammon's findings indicated that Japanese B encephalitis (JBE) virus was active in these cases. Blood specimens drawn on the first and fifteenth day of illness from suspected cases, and frozen brains and cords from the fatal cases, were flown to either the Hooper Foundation or the 406th Medical General Laboratory in Tokyo. These two laboratories collaborated on the virus studies.

The epidemic reached its peak in the middle of January and gradually subsided. The last case entered Guam Memorial Hospital on April 6, 1948. Army investigators from Tokyo and civilian investigators from the Hooper Foundation arrived here when the epidemic was declining.

The epidemic lasted about 4 months. Forty-six cases occurred among natives and eight among Navy personnel. No cases were seen at the Army Hospital on Guam. During the following 8 months only two mild cases of encephalitis occurred.

The patients are divided into four etiological groups on the basis of the results of the virus studies. Hammon *et al.*, (1958) have reported in greater detail the results of the virus studies. These groups are summarized in Tables 1, 2, 3, and 4. The basis for placing a patient in a certain group is as follows:

I. *Japanese B encephalitis* was diagnosed if there was a significant rise in titer of the

[1] The material for this communication was collected while the authors were on active duty as Lt.(jg) MCR USNR at Guam Memorial Hospital and U. S. Naval Hospital, U. S. Naval Medical Center, Guam, M. I.

[2] The views expressed here are not necessarily those of the Bureau of Medicine and Surgery, U. S. Navy.

TABLE 1

*Patients with Japanese B encephalitis**

Case No.	Onset	Discharged	Age	Sex	Recent mumps	Mumps Contact	Max. temp.	Headache	Vomiting	Stiff neck	Stupor	Convulsions	Coma	Personality changes	Reflex changes	Days of fever	Days of neuro. signs	Days of lethargy	Spinal fluid WBC	Spinal fluid Prot.
1	12–23–47	1–9–48	11	F	+ (6 wks)	+	104	+	–	+	+	+	+	+	+	9	9	16	78	N
																			46	Up
2	1–1–48	1–15–48	11	F	–	+	105	+	+	+	+	–	–	–	+	6	8	10	77	Up
																			32	N
3	1–5–48	1–18–48	19	M	–	–	102	+	–	+	–	–	–	–	–	1	0	2	220	Up
4	1–6–48	2–3–48	6	M	+ (5½ wks)	+	104	+	+	+	+	–	–	+	+	5	7	9	410	N
5	1–5–48	1–25–48	9	F	–	–	105	+	+	+	+	+	+	+	+	10	11	13	780	Up
6	1–7–48	1–15–48	1	M	–	+	106	+	+	+	+	+	+	+	+	..	died	..	60	N
7	1–7–48	1–20–48	16	M	–	+	104	+	+	+	+	–	–	+	–	6	0	9	70	N
																			100	Up
8	1–10–48	1–25–58	2	M	–	–	102	+	+	+	–	+	–	+	+	8	8	10	100	N
9	1–11–48	1–25–48	3	F	–	–	102	–	+	+	–	–	–	–	+	5	5	10	260	Up
10	1–14–48	1–31–48	1	M	–	–	104	+	+	+	+	+	–	+	+	7	5	9	82	N
11	1–15–48	1–31–48	2	M	–	–	104	–	+	+	+	–	+	+	+	7	6	8	154	N
12	1–23–48	2–15–48	8	M	–	+	105	+	+	+	+	+	+	+	+	7	10	30	310	Up
13	2–3–48	2–10–48	40	F	–	–	105	+	+	–	–	–	–	+	–	1	3	4	80	N
†14	1–10–48	1–19–48	19	M	–	–	107	+	–	+	+	–	+	+	+	..	died	..	220	Up
†15	1–25–48	1–29–48	34	M	–	–	104	+	–	+	–	–	–	+	+	..	died	..	283	Up
†16	2–10–48	3–1–48	23	M	–	+	100	+	–	+	+	–	–	–	+	2	1	2	880	N

* + = yes; – = no.

† Non-Guamanian personnel or Guamanians in U. S. Military Forces who were treated at the U. S Naval Hospital on Guam and were not evaluated by Pieper and Kurland in their 10-year follow-up evaluation (Pieper and Kurland, 1958).

TABLE 2

*Patients with mumps encephalitis**

Case No.	Onset	Discharged	Age	Sex	Recent mumps	Contact	Max. temp.	Headache	Vomiting	Stiff neck	Stupor	Convulsions	Coma	Personality changes	Reflex changes	Days of fever	Days of neuro. sign	Days of lethargy	Spinal fluid WBC	Spinal fluid Prot.
1	12–21–47	12–29–47	19	M	+	+	100	+	–	+	–	–	–	–	+	2	2	4	402	N
2	12–24–47	1–3–48	4	F	+	+	103	+	+	+	–	–	–	–	+	4	3	5	280	N
3	12–25–47	1–3–48	1	F	–	+	105	+	–	+	+	–	–	–	+	6	3	6	42	N
4	1–21–48	2–2–48	23	M	+	+	104	+	+	+	–	–	–	–	+	6	2	2	360	Up
5	2–2–48	2–14–48	12	M	–	+	103	+	+	+	–	–	–	–	–	1–	0	2	208	Up
6	2–5–48	2–15–48	3	M	–	+	102	+	+	+	–	–	–	+	+	2	1	4	280	N
7	2–7–48	2–15–48	19	F	+	+	104	+	+	+	–	–	–	–	+	4	2	1	503	Up
8	2–15–48	3–1–48	2	F	–	+	105	–	+	+	–	–	–	–	+	4	1	3	40	N
9	2–23–48	2–28–48	19	M	+	+	103	+	+	+	–	–	–	–	+	3	3	2	702	Up
10	2–28–48	3–4–48	17	F	+	+	100	+	+	–	–	–	–	–	–	2	0	2	281	Up
11	3–1–48	3–8–48	13	F	–	–	100	+	–	–	–	–	–	–	–	1	0	1	136	N
12	3–5–48	3–12–48	38	F	–	–	100	+	+	–	+	–	–	+	+	1	2	5	27	..
13	3–6–48	3–12–48	9	F	+	+	105	+	+	+	+	–	–	–	+	2	1	1	220	..
14	3–8–48	3–12–48	18	M	+	+	101	+	+	+	–	–	–	–	–	2	0	1	32	..
†15	12–16–47	2–19–48	19	M	+	+	104	+	+	+	–	–	–	–	+	2	1	0	125	Up
†16	2–10–48	2–19–48	26	M	+	+	104	+	+	+	–	–	–	–	+	4	2	3	80	N
†17	2–14–48	2–24–48	35	M	+	+	103	+	–	–	–	–	–	–	–	1	0	1	180	N

*† See footnotes to Table 1.

TABLE 3

*Patients with Japanese B encephalitis and positive serology for mumps**

Case no.	Onset	Discharged	Age	Sex	Recent mumps	Contact	Max. temp.	Headache	Vomiting	Stiff neck	Stupor	Convulsions	Coma	Personality changes	Reflex change	Days of fever	Days of neuro. signs	Days of lethargy	Spinal fluid WBC	Spinal fluid Prot.
1	12-1-47	1-28-48	1	F	+	+	104	−	−	+	+	+	−	+	+	22	18	30	41	N
2	12-17-47	1-3-48	2	M	−	+	104	+	+	+	+	+	−	+	+	6	6	13	47	Up
3	12-23-47	12-29-47	11	M	−	−	100	+	+	+	−	−	−	−	+	1	1	2	382	N
4	12-25-47	1-13-48	6 mo.	F	−	−	104	+	−	+	+	+	−	+	+	7	6	6	35	Up
5	1-6-48	1-21-48	7 mo.	F	−	+	103	+	+	+	+	+	−	+	+	5	5	5	70	N
6	1-8-48	1-12-48	4	M	−	−	106	−	−	+	+	+	+	+	+	..	died	..	10	N
7	1-9-48	1-23-48	7	M	−	+	105	+	+	+	+	+	−	+	+	5	6	9	160	N
8	1-13-48	1-20-48	16	M	+	+	103	+	+	+	−	−	−	−	+	3	3	3	320	N
9	1-11-48	1-21-48	7	M	+	+	105	+	+	+	+	−	−	+	+	3	3	5	260	N
10	1-11-48	1-24-48	15	M	+	+	102	+	+	+	+	−	−	−	+	5	4	4	45	—
11	1-14-48	2-2-48	15	F	−	+	105	+	+	+	+	−	+	+	+	13	15	19	123	Up
12	1-19-48	2-5-48	7	M	−	−	105	+	+	+	+	+	+	+	+	3	4	5	72	N
13	1-24-48	2-11-48	4	M	+	+	103	+	+	+	−	+	−	+	+	2	4	3	49	Up
14	1-29-48	2-2-48	14	F	+	+	103	+	−	−	−	−	−	−	+	2	1	1	2	N
†15	1-19-48	2-10-48	28	M	−	−	104	+	−	+	−	−	−	−	+	5	2	1	52	N

*† See footnotes to Table 1.

TABLE 4

*Patients with encephalitis of unknown etiology**

Case no.	Onset	Discharged	Age	Sex	Recent Mumps	Contact	Max. temp.	Headache	Vomiting	Stiff neck	Stupor	Convulsions	Coma	Personality changes	Reflex changes	Days of fever	Days of neuro. signs	Days of lethargy	Spinal fluid WBC	Spinal fluid Prot.
1	1-7-48	1-16-48	26	M	−	−	103	+	+	+	+	+	−	+	+	6	6	6	60	N
2	1-7-48	1-11-48	4	F	−	+	108	‡	−	+	+	+	+	+	+	‡	died	‡	20	N
3	1-5-48	1-26-48	67	F	−	−	103	+	−	+	+	−	−	+	+	5	4	8	90	..‡
4	1-22-48	2-5-48	3	M	+	+	103	+	+	+	−	+	−	+	+	5	2	2	80	N
5	2-12-48	2-28-48	57	M	−	−	104	+	−	+	+	−	−	+	−	8	2	5	122	Up
†6	12-28-47	2-19-48	19	M	−	−	101	+	−	+	+	−	−	+	+	9	3	52	91	N

*† See footnotes to Table 1.

‡ No data.

neutralizing (N) antibodies and/or complement-fixing (C-F) antibodies in serial blood specimens or if the virus was found at autopsy. Three patients had only one blood specimen examined but in all three there was a very high titer in both the N and C-F antibodies. Also it was required that C-F antibodies for mumps should not show an increase unless there was a history of parotitis having occurred more than 2 weeks before the onset of encephalitis.

II. *Mumps encephalitis* (ME) was diagnosed if the tests for JBE were not as described under I and there was either clinical mumps at a suitable interval (2 weeks) or rising titer of C-F antibodies.

III. *Japanese B encephalitis with mumps* was diagnosed if the tests for JBE were indicative of current infection and if there was either recent (2 weeks) clinical mumps or rise in titer of the C-F test for mumps.

IV. *Encephalitis of unknown etiology* (EUE)

was diagnosed if none of the criteria described above was met and if specific tests for certain other known causes of encephalitis, e.g., western equine and St. Louis viruses were negative. An adequate number of specimens for determining an antibody-titer rise was not available from most of the cases included in this group.

The reader is referred to larger series of cases for complete description of the symptomatology and findings of JBE (Lewis *et al.*, 1947) and ME (Symposium, 1940). Since the numbers of cases in our group are small, we shall comment only on the more prominent differences.

SYMPTOMATOLOGY AND PHYSICAL FINDINGS

The onset of the disease was usually sudden in cases of JBE and mixed groups, while it often began gradually during, or shortly after, the acute stage of mumps infection in cases of pure ME.

Almost all of the cases in each group complained of headache, vomiting, and fever. There were no significant variations in the frequency of these findings, but increased severity and duration were prominent among patients with JBE. Stupor was unusual in ME but common in other groups. Coma and convulsions were fairly frequent in cases with JBE and mixed infections but were absent in ME.

Stiff neck and positive Kernig's sign were seen in almost all patients but lasted longer in cases of JBE infection. Only two patients had a flaccid paralysis and both of these had JBE. In general, all patients had diminished reflexes. There were no other prominent findings except those occurring during or after convulsions, such as spastic paralysis, clonic and tonic contractions, and disturbances of the extraocular muscles. No patient with pure ME had convulsions or was in coma.

In summary, JBE may be strongly suspected if the onset is sudden, if the headache and vomiting are severe and of long duration, if neurological signs are prominent, and if coma and convulsions are present.

LABORATORY FINDINGS

There were no essential differences between the white blood counts or differential counts of the two groups. In almost all patients the white blood count was moderately elevated, with a shift to the left.

Examination of the spinal fluid did not serve to differentiate the diseases. The findings were similar in each group.

The spinal fluid pressure was usually slightly, but rarely greatly, elevated. The spinal fluid protein was moderately elevated in about half the cases of all groups. The sugar and chloride were normal. In all cases the spinal fluid contained an excess of white blood cells, although on occasion the number of cells was normal in the final spinal tap. The predominant cell in almost all spinal fluids was a large mononuclear cell, at times indistinguishable from a polymorphonuclear cell. In a few of the cases lymphocytes or polymorphonuclears predominated but no correlation could be found between the cell count or cell type and the final diagnosis.

MORTALITY AND SEQUELAE

Four of the five patients who died had JBE. One of these also had positive C-F for mumps. The virus of JBE was isolated from these four patients from autopsy material. The fifth patient had N antibodies to JBE virus in the only blood specimen collected (fourth day), plus typical pathological findings of JBE. No virus was isolated from the brain and, with only one blood specimen available, the required diagnostic criteria were not met.

All patients were checked for postencephalitic changes, 3, 6, and 10 months after discharge. Four patients had severe symptoms of mental deterioration and personality change at discharge, but at the final examination all seemed to be normal. One of these had EUE, while two had JBE type, and one a mixed infection.

REPORT OF CASES

The following case reports are submitted to illustrate the various manifestations of the encephalitis epidemic.

Case I (Table 3, No. 11) *JBE with positive serological tests for mumps.* A 15-year-old Guamanian girl, admitted to the hospital on January 15, 1948 had complained of sudden onset of fever, headache, vomiting, and pain in the neck for 1 day. The patient had had recent contact with mumps but had not contracted the disease.

Physical examination revealed a well-developed and well-nourished girl who was very restless, irrational, and uncooperative. The pupils reacted

to light. Extraocular movements could not be tested because of inability to focus adequately. There was no deviation of the eyes, paralysis, or weakness. The neck was very stiff with pain on flexion. Kernig's and Brudzinski's signs were positive. The plantar reflexes were normal. The deep reflexes were hypoactive throughout. Temperature was 104.6°F.

Spinal tap on admission showed a pressure of 240 mm of water and was ground-glass in appearance; 37 red blood cells (rbc) and 127 white blood cells (wbc) were present, 73 per cent being large mononuclears. The protein, sugar, and chloride were normal. Smear and culture were negative. Repeat spinal tap 8 days after admission revealed no cells and normal total protein.

Several hours after admission she became comatose but responded to painful stimuli. The reflexes remained unchanged. A day later her temperature was 105.4°. She was no longer comatose, but would not cooperate. She continually voided and defecated in bed. Five days after admission the temperature was still over 103° at which time she responded rationally to questions but continued to act irrationally. Kernig's and Brudzinski's signs were negative but a slight nuchal rigidity persisted. She continued to improve and 13 days after admission showed only a low-grade fever, anorexia, and severe lethargy. These findings gradually subsided and she was discharged on her nineteenth hospital day. At this time she had no neurological abnormalities or evidence of mental deterioration, but showed slight weight loss and slight lethargy.

Her parents brought her back to the hospital March 10, 1948 because of abnormal behavior and personality changes. They stated that she had been perfectly well at home for 2 weeks and then began to act "funny." Examination on this date showed her to have no pathological reflex changes but she was obviously not normal mentally. She grinned constantly and had delusions and poor judgment. Memory for recent and past events was poor. At times she could remember events but misplaced them as to time and sequence. Immediate treatment consisted of sedation with no favorable response. On April 2, 1948 she was started on subshock insulin therapy which was discontinued after 2 weeks. She was discharged on the next day with no psychiatric manifestation.

She was seen again in August, 1948 and was normal except for a complete lack of moral sense. She was seen again in October, 1948 and seemed perfectly normal except for amnesia covering a 3-month period during and after her first hospitalization. Her sisters and mother said that she very seldom left the house and was very helpful with the household duties. They considered her normal and we agreed.

Serum drawn on the day after admission and 14 and 27 days later showed a rise of the C-F titer and N index for JBE. The serum was positive throughout for C-F antibodies for mumps at a dilution of 1:64.

Case II (Table 2, No. 10) *ME*. A 17-year-old Guamanian girl was admitted February 28, 1948 with complaints of headache, vomiting, and fever of 2 days' duration. Her symptoms were thought to be hysterical in origin because of a recent scolding by her father. System review showed that she had bilateral foot drop of 3 years duration, present also in her older sister.

Physical examination showed her to be a cooperative, intelligent girl who complained bitterly of severe generalized headache. The temperature was 100°F. She had no stiff neck or other findings of encephalitis except vertigo and hyperkinesia. Incidental findings were a complete bilateral foot drop, decreased knee jerks, and absent ankle jerks, all of which had been present for 3 years. Sensation, position, and vibratory sense were normal.

The spinal fluid contained 52 rbc and 281 wbc, of which 89 per cent were large mononuclears and 11 per cent lymphocytes. Other laboratory data were not unusual.

The spinal tap relieved her headache for 3 hours. The day after admission her temperature was normal and she had no positive findings of encephalitis and no complaints. Six days after admission she had a rise in temperature to 100°F, bilateral painful swelling over a parotid area, and injection of Stenson's duct on the left. This was considered to be mumps. It cleared completely in 5 days.

Sera drawn on the first, fourteenth, and twenty-eighth days of illness gave negative C-F reactions and N index for JBE. She was seen three times in the following 9 months and showed no postencephalitic change. Her chronic neurological disease was diagnosed as muscular atrophy of the distal type.

Case III (Table 4, No. 2). *EUE, but probably JBE, acute, with fatal outcome.* A 4-year-old female was admitted to Guam Memorial Hospital on January 8, 1948 with the chief complaint of convulsions. On the previous day she had had fever and one convulsion, but felt fairly well the rest of the day except for the fever. On the morning of admission she had another convulsion and was brought to the hospital. Further history revealed that her brother had mumps the previous week.

Physical examination showed a 4-year-old Guamanian female who was comatose. The eyes rolled back and forth continually but were held

most often to the left. The pupils reacted to light. Her extremities showed slight spasticity on the left, with normal reflexes. The neck was slightly stiff. A positive Kernig's sign was present. Plantar responses were normal.

The spinal fluid showed 4 rbc and 20 wbc, 65 per cent of which were polymorphonuclear and 35 per cent lymphocytes. The white blood count was 18,500, with a normal differential. The red blood count was 3,000,000. The stool contained hookworm ova.

Throughout the patient's stay she was comatose and had two convulsions. On the second day in the hospital the eyes deviated to the left with left lateral nystagmus. The head was pulled to the left and the mouth drooped on the left side. These findings continued until death, 3 days after admission. Terminally, she developed fever of 110°F, Cheyne-Stokes respirations, and inability to cough up excess mucus in her throat.

Blood drawn the day before death gave a negative C-F reaction and positive N index (580) for JBE. There were no C-F antibodies for mumps. No virus could be isolated from her brain stem by Dr. Hammon.

Case IV (Table 1, No. 16) *Encephalitis, acute, which simulated a bulbar type of Poliomyelitis.* This 34-year-old civilian worker was admitted to the U. S. Naval Hospital on January 27, 1948 with complaints of fever, chills, and sweats. For the 2 previous days he had had malaise, headache, and body aches. He had had similar attacks previously and was told that they were due to malaria. Atabrine had always brought relief.

"Physical examination revealed him to be covered with a blanket and having a chill. Remainder of examination was negative." A blood smear was negative for malaria.

The next day the patient still had a temperature of 104° F and developed vomiting. On the second hospital day he experienced visual hallucinations. Examination showed the facial movements and speech to be uncoordinated, and an incomplete flaccid paralysis of both upper extremities. The neck muscles were weak; there was no headache or stiff neck. A few hours later the patient could not move his right shoulder or entire left upper extremity. He was still free of headaches. There was now moderate nuchal rigidity. Left biceps, triceps, and supinator reflexes were absent. Spinal puncture at this time showed 283 wbc with 60 per cent polymorphonuclears. The protein and sugar were normal.

Later on the same day he showed a clear sensorium, flaccid paralysis of the left upper extremity, and some difficulty in swallowing.

Early the next day the patient stated that he felt better, but still showed paralysis. Fifteen minutes later he expired quietly and unexpectedly. Autopsy showed slight softening, edema, and congestion of the brain and spinal cord, and acute pulmonary edema. JBE virus was isolated from the brain by the 406th Medical General Laboratory in Tokyo. The autopsy findings were indistinguishable from those of poliomyelitis.

PATHOLOGY

The pathological description is comparatively brief because of the nature and length of the present article.

Central Nervous System

All five fatal cases were autopsied. Brains and spinal cords were removed in all instances. A total of 41 blocks taken from brain and spinal cords, quite equally divided among the five cases, were made available to the authors. All sections were fixed in formalin or Zenker's solution and stained with hematoxylin and eosin.

Gross Description

The gross findings were not remarkable. In all cases the leptomeninges were clear and of normal thickness, and revealed no evidence of exudate or hemorrhage. However, clear fluid was present in moderate amounts in three cases, and congestion in all instances. The brains and spinal cords were of normal to slightly increased size, and of normal to slightly softened consistency.

Microscopic Description

Findings were quite similar in all cases. The inflammatory cells consisted almost entirely of lymphocytes, microglia, histiocytes, and "mononuclears." The latter were extremely numerous. The size of their nuclei was approximately equal to, or slightly smaller than, that of a histiocyte. Its nuclear membrane and chromatin stained somewhat more heavily. The shape of their nuclei varied greatly. Many were oval. Most, however, were indented, club- or teardrop-shaped, or mimicked the letters "L," "Y," and "U." Cytoplasm was practically absent. These cells were found in all cases and constituted the most common inflammatory cells outside of the leptomeninges and immediate perivascular areas. They were the principal cells of the "glial nodule." The term "mononuclear" used here refers to this type of cell alone, and is employed to avoid interpretation as to its origin.

No polymorphonuclear leukocytes were seen. A few foci of gitter cells were present. Lymphocytes were restricted almost exclusively to the leptomeninges and perivascular regions. Histiocytes were uncommon and tended to be associated with lymphocytes. Oligodendroglia and astrocytes were very conspicuous. These latter two types of cells were much less numerous than microglia. All three, when present, were usually associated with mononuclears. Such cellular groupings were labeled "glial nodules." Gliosis, fibrosis, thrombosis, and calcification were not seen.

Leptomeninges, Vessels, Spinal Canal, Ventricles. The leptomeninges were absent over much of the tissue examined. The findings were everywhere slight. A few lymphocytes and histiocytes, as well as an occasional mononuclear and uncommon microglia, were seen. Vessels were congested in most areas.

The vessels within the parenchyma were generally congested. Perivascular "cuffing" by inflammatory cells was the rule in all cases and varied in intensity. Lymphocytes predominated, but a few mononuclears and histiocytes and a rare microglia were also seen. Perivascular hemorrhages were present only in the pons of one case. This case was also the only example showing perivascular edema.

The spinal canal and ventricles of the brain were not remarkable except for slight dilatation of the former in one microscopic section.

General Description of Parenchymal Lesions. Slight to moderate numbers of nuerons, for the great part motor, showed mild to moderate degeneration; small numbers appeared destroyed. Lesions were most common in the spinal cords and brain stem, and least pronounced in the cerebral cortex.

Associated with damaged neurons were found accumulations of mononuclears and microglia, occasional oligodenroglia, astrocytes and lymphocytes. These foci of cells were common throughout, and could be seen with low-power magnifications, usually as round, oval or scattered groups of fairly dark nuclei. They frequently surrounded or replaced destroyed or degenerating neurons, most commonly anterior horn cells. These aggregations of cells are referred to as "glial nodules" and strongly resemble similarly-named lesions described in the literature.

The glial nodules were found chiefly in the gray matter. However, they were fairly numerous in the white matter of both brain and spinal cord, although rarely equaling in frequency lesions of gray matter. An occasional dorsal column of the spinal cord was involved.

Description of Anatomical Location

Spinal cord. All segments of the spinal cord were diseased, although the intensity of the process varied somewhat from case to case and among different levels of a given spinal cord. Perivascular cuffing, chiefly by lymphocytes, was the rule. Degeneration of anterior horn cells was quite common; death of cells less frequent. These cells were frequently decreased in number. Glial nodules surrounded or replaced diseased neurons. The white matter revealed glial nodules as well as a few areas which stained slightly, showed disrupted architecture and fewer axons, and contained a few mononuclears and microglia. In one area a wide ribbon of mononuclears and microglia bordered the ventral periphery of the cord on one side. Now and then a dorsal column contained glial nodules.

Brain stem (pons, medulla oblongata). The lesions were generally somewhat less prominent than those of the spinal cord, although equaling the latter in areas. Perivascular cuffing was present in all cases. Mild perivascular hemorrhages and edema were found in the pons of one case. Glial nodules existed in small-to-moderate numbers and were frequently associated with small foci of degenerating, and occasionally destroyed, neurons. Similar cells were also found in the white matter. A few light-staining areas of destruction similar to those described under "spinal cord" were seen in both gray and white matter. These foci contained gitter cells in the one pons which was the seat of perivascular hemorrhages and edema.

The cerebellum was represented by but one small section from each of two cases. In one no abnormalities were found. In the other, perivascular cuffing was absent, and neurons appeared intact. However, a few small glial nodules were present, primarily near neurons.

Cerebral cortex. Neurons were generally well preserved. A few revealed mild degeneration. Glial nodules were quite numerous and varied in size. Several light-staining foci of disrupted architecture, with slight-to-moderate numbers of inflammatory cells, chiefly mononuclears and microglia, were present. One such lesion contained gitter cells. Such areas, as well as glial

nodules, were present in both gray and white matter, primarily the former.

COMMENT

Pathologically, all cases resembled each other closely. The relatively severe involvement of the spinal cord, usually exceeding that of the cerebral cortex, is of interest. These changes resemble closely those seen in poliomyelitis, as well as in St. Louis and equine encephalitis. Nevertheless, only one case (Table 1, No. 16) presented a clinical picture indistinguishable from poliomyelitis. The remaining four fatal cases did not resemble this disease clinically yet microscopically showed changes consistent with those found in poliomyelitis. The similarity of microscopic findings in the disease mentioned extends into the brain proper but the topographic distribution was different in the cerebral cortex. For example, lesions in the cortex, except in the motor area, are seldom seen in poliomyelitis while in JBE a diffuse cortical involvement is frequently seen.

The origin of the "mononuclear" cells is unknown. They may be hematogenous, derived from the central nervous system, or from both.

The location in the white matter of both "glial nodules" and light-staining disrupted areas is not described in the literature on JBE as far as the present writers are aware (Zimmerman, 1946; Haymaker and Sabin, 1947).

Pathological findings outside the central nervous system bore no specific relationship to the encephalomyelitis with the possible exception of pulmonary hemorrhage and pulmonary edema; no lesions suggestive of virus etiology were seen.

DISCUSSION

Epidemic encephalitis is characterized by its appearance in epidemic form, by acute onset, and by signs of meningeal irritation and disease of the encephalon. The main symptoms, headache and fever, are those of meningeal irritation, disturbances in consciousness, and those due to focal brain lesions.

The differentiation from poliomyelitis may occasionally be difficult. Poliomyelitis is usually manifested mainly by anterior-horn-cell disease; encephalitis by disease of the higher centers. However, the picture may sometimes be reversed. One of our patients looked and acted like a typical case of poliomyelitis (Table 1, No. 16, Case Report IV) and yet yielded the virus of JBE at autopsy.

Bacterial meningitis may be differentiated by typical spinal-fluid-chemistry changes and the finding of the causative organism.

The clinical diagnosis of virus encephalitis may be made quite easily. However, the diagnosis of the specific virus is difficult. Virus encephalitis may be divided into two groups; those spread by arthropods and those not spread by arthropods. The arthropod-borne group (such as JBE, Western equine, St. Louis virus) usually occur in epidemics and can be differentiated only by extensive and time-consuming tests such as C-F, N index, and virus isolation. Specific diagnosis as to type probably cannot be made by clinical means alone. The nonarthropod-borne group (such as postinfectious and postvaccination encephalitides) can usually be differentiated by clinical means. They occur sporadically, offer a history which suggests a type, and usually present a fairly mild clinical picture.

The differentiation of the postinfectious group from the arthropod-borne group appears easy but may be difficult. Thus, as in one case (Table 3, No. 14) the patient was just recovering from mumps and came down with a mild encephalitis which would be presumed to be due to mumps, and yet serological work showed infection with JBE virus also. Again, in another case (Table 2, No. 3) we may have no preceding mumps infection, yet severe disease such as occurs in JBE, while the sera may be negative for JBE but positive for mumps.

Along with the epidemic of encephalitis there were present at least 1,500 cases of mumps. This led us to anticipate at least 10 cases of mumps encephalitis and at first we felt that our cases were all due to the virus of mumps. JBE virus was isolated from four of the patients who died, and many others showed a significant rise in C-F antibody and N index. Therefore, we may state that JBE virus infected people on Guam, caused clinical evidence of infection and was the probable cause of death in five individuals. Seventeen other individuals had presumptive evidence of a meningeal reaction due to mumps virus, with no evidence of infection

with JBE virus. Hence, there were two types of encephalitis on Guam at the same time.

Further analysis of the virus studies shows that there is even more overlapping. Thus, 16 patients had JBE alone, 17 patients had ME alone, but six of these did not have clinical parotitis. Of the 15 patients with both JBE and mumps infection, nine did not have clinical parotitis. It is apparent that 60 per cent of our cases of mumps infection were not accompanied by parotitis, orchitis, etc. That this also occurs frequently in the United States is attested to by reports in the literature (Henle and McDougall, 1947; Eberleim and Lynxweiler, 1947).

It was also noted that five (25%) of the mild cases were due to JBE virus alone, two (16%) were due to a mixed infection, and ten (60%) were due to mumps virus alone.

The above statements suggest two questions: (1) why did encephalitis due to JBE virus occur at this time? (2) was there any relationship between it and the epidemic mumps?

The virus of mumps may have served to convert the inapparent JBE infections into overt clinical infections. Some workers believe that this may happen (Birnberg, 1935; Gunderson, 1927). However this could not have been a factor in those patients who had JBE with no clinical or serological evidence of the presence of mumps virus. We can only speculate on the possible role of the mumps virus in the mixed infections. Can the one virus potentiate the action of another?

In general the epidemic was quite mild. Analysis of those cases with Japanese B alone showed that they were no more severe than the mixed infections. The cases of "pure ME" were the mildest of all but several Japanese B infections were just as mild. One of the mumps infections was as severe as three fourths of the Japanese B infections. Therefore, the mildness of the disease in an individual patient is no indication of etiology in a mixed encephalitis epidemic. Emphasis should be placed on the fact that the etiology in an individual case of mixed encephalitis cannot regularly be diagnosed clinically even with a suggestive history.

SUMMARY

This is a report of an encephalitis epidemic which occurred on Guam in late 1947 and early 1948. The virus of Japanese B encephalitis was isolated from four of the five fatal cases and serological tests indicated that it was the etiological agent in many others. Concurrent with this epidemic there was an epidemic of mumps with several cases of encephalitis due to this virus. Other patients showed infection with both viruses and in others no identification of the exact virus was accomplished.

These four groups are compared and discussed individually in relation to symptoms, signs, and laboratory findings, and four cases are presented in detail. The pathological findings are presented and emphasis is placed on one patient who clinically and pathologically had a disease indistinguishable from poliomyelitis but in whom the virus of Japanese B encephalitis was isolated. Further stress is laid on the exact diagnosis of the virus present by use of the proper virus studies.

CONCLUSIONS

1. The exact etiology of individual cases of central-nervous-system virus infections cannot be diagnosed clinically with any degree of accuracy.

2. Japanese B encephalitis may occur as far east as Guam and should be considered in areas more distant from Japan.

3. Mumps infection frequently occurs with no parotitis.

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SEQUELAE OF JAPANESE B AND MUMPS ENCEPHALITIS

Recent Follow-Up of Patients Affected in 1947–1948 Epidemic on Guam

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The reported frequency and severity of sequelae of acute infectious encephalitis depend upon numerous variables, including the type and virulence of the organism, the availability and utilization of medical care by the affected population, and the adequacy of the later examinations. The careful attention to detail in clinical diagnosis and serological confirmation described in the preceding papers of this series, (Edgren *et al.*, 1958; Hammon *et al.*, 1958) and the residence stability of the native population on Guam have afforded an unusual opportunity for a long-term follow-up of the patients with encephalitis who were affected during the 1947–1948 epidemic. In the present report we shall consider the frequency and characteristics of sequelae and the prognostic value of several clinical features of the acute illness as observed among the Guamanian civilians treated at Guam Memorial Hospital.

The preceding reports in this series suggest that almost all civilians with moderate or severe clinical encephalitis were hospitalized and received the best available care at the Guam Memorial Hospital which, at that time, was operated by personnel of the U. S. N. Medical Department. However, on the basis of serological evidence, these clinical cases represent only a small fraction of the total infected in the population (Hammon, *et al.*, 1958). It is likely that, aside from inapparent infections, those with very mild illness did not seek admission to the hospital. The case fatality ratio and the proportion of patients developing sequelae as described in this report were also influenced by the ready acceptance by the native population of the fine hospital care available to them.

METHODS

A list of 54 encephalitis patients described in the preceding papers was furnished us by Drs. Edgren and Hammon (*loc. cit.*). With this list we were able to identify the 46 civilian patients treated at Guam Memorial Hospital. The hospital record for the acute illness, and all subsequent admissions to the hospital or outpatient department, were reviewed for each of these 46 patients. With the assistance of the local public health nurses, the latest residence of each of the 43 survivors was determined. The 32 patients still residing on Guam were interviewed and examined on one or more occasions between March and September, 1957, almost 10 years after the epidemic.

The standard neurological history and examination form of the Mayo Clinic was used as a guide to insure a complete systematic investigation of each patient (Mayo Clinic, 1956). The psychological and intellectual status of each patient was also judged on the basis of a history of his behavior and social adjustment in the community, and on reports from the village nurse, the patient's family and, where indicated, from the school authorities or the village commissioner, or scholastic work and achievement.

The other 11 survivors, according to the village nurses, were apparently in excellent health and most had left the Island in the last 2 or 3 years. Five were males: four had recently joined the U. S. Armed Forces, one was attending college in Ohio. One female, a native of the Island of Palau, had returned to her home. The five other females had moved to the Continental United States with their husbands or parents. For the purpose of the study, these 11 survivors were considered normal in the absence of evidence to the contrary. In the course of the study it was possible to obtain a detailed neurological and psychiatric examination report for each of the men in the Armed Forces and for two others in the United States. These reports confirmed the impression of an excellent recovery from encephalitis in each of these persons.

RESULTS

Japanese B Encephalitis (JBE). In this group there were 17 males and 10 females. Of these,

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TABLE 1

Japanese B encephalitis sequelae

Number*	Initials	Years age at onset	Sex	Died	Types of sequelae: None	Neurological	Intelligence	Psychiatric complaints	No detailed follow-up	Comments
I-1	D, R	11	F	–	–	–	–	+	–	Occasional "black-out spells;" repetitive auditory and visual hallucination in left peripheral field; frequent attacks of unexplained nausea and vomiting.
I-2	Q, A	11	F	–	–	–	–	–	+	Living in U.S.A., presumably well.
I-3	G, NC	19	M	–	+	–	–	–	–	Retarded deaf-mute from birth. Has bilateral Babinski sign. Considered 'No change' due to encephalitis.
I-4	S, J	6	M	–	+	–	–	–	–	Normal.
I-5	F, M	9	F	–	–	+	+	–	–	Moderately confused and retarded. Traced figures and 2-point discrimination inadequate. Neurological exam. otherwise negative. Uncertain whether poor response on intellectual basis or poor sensory association.
I-6	A, JM	8/12	M	+						
I-7	LG, E	16	M	–	+	–	–	–	–	In U. S. Army.
I-8	J, J	2	M	–	–	+	–	–	–	History of intermittent vertigo, diplopia, "black spells;" left lateral nystagmus, bilateral sucking reflex and Babinski sign.
I-9	A, R	3	F	–	+	–	–	–	–	Normal.
I-10	Q, V	1	M	–	–	–	–	+	–	Stutters, occasional enuresis. Neurological and intellectual tests normal. Related to encephalitis?
I-11	M, J	2	M	–	+	–	–	–	–	Normal.
I-12	G, HR	8	M	–	+	–	–	–	–	Normal.
I-13	A, N	40	F	–	+	–	–	–	–	Normal.

* Numbers refer to and correspond with Table (Roman numeral) and case (Arabic numeral) in previous paper in this seris by Edgren *et al.*, (1958).

13 had had JBE and 14 were considered to have had mumps infection concurrently with, or just prior to, the onset of JBE. No difference between the frequencies of occurrence of sequelae in these two groups (with or without mumps) was noted, and there was no appreciable difference in the sequelae between the two sexes. (Tables 1 and 1A)

In this group, two children under 5 years of age died. Of the remaining 25 patients, follow-up examinations were completed on 23. Two young ladies, ages 20 and 23 years, who were living in the continental United States were not examined but, as mentioned above, will be considered as having no sequelae.

Fifteen of the 25 are considered as having suffered no sequelae of the encephalitis. Two of the 15 may have been injured or predisposed to further brain injury but circumstances made it difficult to evaluate the degree of brain injury which might have resulted from the encephalitis. One of the two was a deaf mute, mentally-retarded child who was found to have bilateral extensor plantar responses; he was said to be no worse after the encephalitis (I-3)[3]. The other had apparently recovered from encephalitis but had had a head injury in 1953 which was followed by grand mal seizures and intellectual difficulties (III-2).

In the remaining ten patients, eight had

[3] Numbers refer to, and correspond with, numbers in the previous report in this series by Edgren *et al.*, (1958). Roman numerals refer to Table number, Arabic numerals refer to patient number.

TABLE 1-A

Mumps plus Japanese B encephalitis sequelae

Number*	Initials	Years age at onset	Sex	Died	Types of Sequelae					Comments
					None	Neurological	Intelligence	Psychiatric complaints	No detailed follow-up	
III-1	T, R T, RT	1	F	–	+	–	–	–	–	Normal.
III-2	T, D	2	M	–	+	–	–	–	–	Presumably normal until 1953. Head injury in 1955 followed after 1 month by daily grand mal seizures, retardation; bilateral Babinski.
III-3	B, PU	11	M	–	+	–	–	–	–	In U. S. Army.
III-4	SN, R	6/12	F	–	–	+	+	–	–	Severely retarded. Right lateral strabismus with amblyopia ex anopsia. Mild spasticity, in all limbs. Occasional enuresis. Bilateral apraxia.
III-5	B/VJ	7/12	F	–	–	+	–	–	–	Postencephalitis febrile seizures until 1954. Left arm weak, slightly atrophied, spastic. Astereognosia, and impaired 2-point discrimination on left. Frequently enuretic.
III-6	D, M	4	M	+						
III-7	SN, Juan	7	M	–	–	–	+	–	–	Mild mental retardation. Neurologically O.K. Poor memory, caculation, recall, general information.
III-8	SN, Jose	16	M	–	+	–	–	–	–	In U. S. Army.
III-9	C, V	7	M	–	–	+	–	–	–	Bilateral Babinski sign only abnormality.
III-10	A, J	15	M	–	+	–	–	–	–	Normal.
III-11	L, B	15	F	–	+	–	–	–	–	Normal.
III-12	C, JF	7	M	–	–	+	+	–	–	Moderate mental retardation, poor memory and recall. Bilateral Babinski, hyperactive reflexes.
III-13	D, F	4	M	–	–	+	–	–	–	Hyperactive deep tendon reflexes all extremities. Bilateral transient ankle clonus and positive Babinski.
III-14	S, M	14	F	–	–	–	–	–	+	Living in U.S.A., presumably well.

* See footnote to Table 1.

neurological or intellectual defects and two had psychiatric complaints. In one patient there was evidence of brain stem and cerebellar dysfunction (I-8). One patient had convulsive seizures, left hemiparesis, and cortical sensory impairment, as demonstrated by moderate astereognosis, poor 2-point discrimination, and weakness, spasticity, and slight atrophy of the left upper extremity (III-5). Three patients had negative examinations except for bilateral extensor plantar responses (III-9, III-13), although one (III-12) was somewhat subnormal on mental testing.

Three other patients were mentally retarded: one was severely retarded and had a moderate spasticity of all limbs and a strabismus due to right lateral rectus muscle weakness (III-4); another patient was moderately retarded and responded poorly to traced figure and 2-point discrimination tests (I-5); the third had poor memory, recall, general information, and ability to calculate (III-7).

Two patients had symptoms suggestive of psychological dysfunction and had been included as having "sequelae." One stuttered and was enuretic, whether related to the encephalitis

TABLE 2

Mumps encephalitis sequelae

Number*	Initials	Age at onset	Sex	Died	Types of sequelae					Comments
					None	Neurological	Intelligence	Psychiatric complaints	No detailed follow-up	
II-1	C, F	19	M	–	–	+	–	–	–	Minimal dysdiadochokinesia on knee pat and questionable error on traced figure responses.
II-2	A, W	4	F	–	–	–	–	–	+	Moved to U.S.A., apparently well.
II-3	Q, L (C, LQ)	1	F	–	+	–	–	–	–	Normal.
II-4	T, I	23	M	–	–	+	–	–	–	Bilateral Babinski only abnormality.
II-5	S, J	12	M	–	+	–	–	–	–	In college in U.S.A., negative exam.
II-6	G, J	3	M	–	+	–	–	–	–	Normal.
II-7	M, J	19	F	–	–	–	–	–	+	Returned to Palau, apparently well.
II-8	B, E	2	F	–	+	–	–	–	–	Follow-up courtesy family physician.
II-9	S, T	19	M	–	+	–	–	–	–	In U. S. Navy.
II-10	T, R (M, RT)	17	F	–	+	–	–	–	–	Charcot-Marie-Tooth syndrome, onset 1946, otherwise normal.
II-11	S, E	13	F	–	–	–	–	–	+	Living in U.S.A., apparently well.
II-12	D, F	38	F	–	–	–	–	–	+	Normal.
II-13	A, M	9	F	–	+	–	–	–	–	Normal.
II-14	F, H	18	M	–	–	+	–	–	–	Bilateral Babinski on present examination.

* See footnote to Table 1.

or not is uncertain (I-10); one other patient (I-1) had complaints more suggestive of organic damage—she had occasional "blackout spells" and repetitive auditory and visual hallucinations, the latter predominantly in the left peripheral field. This patient also complained of poor memory and recall and frequent attacks of unexplained nausea and vomiting.

Mumps Encephalitis (ME). All of the 14 patients who were hospitalized during the epidemic period survived the acute illness. Eight of these were observed during the follow-up study, and correspondence with the military or the family physicians revealed that three others are without sequelae; the remaining three were not examined but are known to be living and, on the basis of information available, are presumed to be well (Table 2).

In eight of the 11 who were examined there were no sequelae. The other three patients had no complaints, but on examination one was found to have a minimal-dysdiadochokinesis (II-1) and the other two had only bilateral extensor plantar responses (II-4, II-14). These three patients were 18, 19, and 23 years of age when affected; their clinical course did not seem to differ from that of the other patients in whom mumps encephalitis was diagnosed. In all three of these patients the diagnosis was based on a concurrent parotitis or recent history of parotitis and the absence of confirmatory serological findings for Japanese B infection. No tests were made for mumps during the acute illness of these patients, and later tests in two were negative for mumps antibodies.

Encephalitis—Type Unknown. Five patients were classified as "encephalitis, type unknown." One patient (IV-2), age 4 years, died during the acute stage of encephalitis. Virus isolation was not successful, although clinically the course was suggestive of JBE. Of the other four patients, examination revealed some neurological and psychiatric dysfunction in all at the time of follow-up study. One patient (IV-1), age 26 years at onset of symptoms, was said to have been mentally retarded prior to the encephalitis and was found to be severely retarded, somewhat dysarthric, and had slight incoordination on knee-patting testing. It is uncertain how much of his dysfunction was due to pre-existing brain

damage. One other patient, age 3 years at onset (IV-4), was thought to be mentally retarded and responded poorly to tests for cortical association function (2-point and traced-figure tests) but how much of this poor response was due to general intellectual deficiency is uncertain. The other two patients were found to have evidence of unstable personalities (IV-3, IV-5) and to have hallucinations. One of these (IV-3) also had transient diplopia, complained of poor memory, and had a bilateral extensor plantar response; the other (IV-5) was said to be recalcitrant, was actively hallucinating (auditory and visual) during the examination, and coordinated poorly on such tests as the knee-pat.

PROGNOSTIC VALUE OF SELECTED CLINICAL FEATURES

Japanese B Encephalitis. There was no appreciable difference in frequency or severity of sequelae between sexes, or in the presence of a concurrent infection with mumps.

TABLE 3

Frequency of sequelae after Japanese B encephalitis, by age of onset

Age, in years, at onset	Total number	Died during acute illness	No detailed follow-up	No sequelae	Neurological and intellectual sequelae	Psychiatric complaints or symptoms
0–4	11	2	0	4	4	1
5–9	6	0	0	2	4	0
10–19	9	0	2	6	0	1
20 plus	1	0	0	1	0	0
Total..	27	2	2	13	8	2

TABLE 4

Frequency of sequelae after mumps encephalitis, by age of onset

Age, in years, at onset	Total number	No sequelae	Neurological sequelae	No detailed follow-up
0–4	4	3	0	1
5–9	1	1	0	0
10–19	7	3	2	2
20 plus	2	1	1	0
Total.....	14	8	3	3

For the encephalitis, *type unknown*, there are too few data to evaluate prognosis by age or clinical characteristics.

The number of cases in each age group is small, but there is a suggestion that sequelae are more severe when the onset occurs in early childhood (Table 3).

Eleven of 17 affected in the first decade died or survived and had sequelae; only one of the 8 who were affected after age 10 years and who could be followed was thought to have some evidence of residual brain damage. The two patients with severe sequelae (III-4, III-5) were 6 months and 7 months of age, respectively, at time of onset.

For the group as a whole, the case fatality was 7.4 per cent; 40 per cent of the survivors had sequelae (11 per cent severe, 29 per cent mild).

Mumps Encephalitis. In this group, there were three patients with sequelae, all males.

The data in Table 4 are too few to be conclusive but suggest a higher incidence of sequelae with increasing age, in contrast to that noted in the preceding tables.

CORRELATION OF CLINICAL FEATURES OF ACUTE ENCEPHALITIS WITH SEQUELAE

In attempting to find an association between clinical course of the acute illness and subsequent development of sequelae, comparisons were made with the tables of clinical data provided by Edgren *et al.*, (1958) and the present status of the patients. No correlation can be found between development of sequelae and the criteria of maximum temperature, headache, vomiting, stiff neck, days of fever, days of neurological signs, days of lethargy, or spinal fluid changes. However, when those criteria which more clearly represent actual neuronal involvement are examined, a correlation is found in those patients who had JBE alone or in conjunction with mumps (Table 5).

In this table the type of encephalitis and the number of patients with and without sequelae are shown in the column to the left. There is no appreciable difference between the JBE cases with, and those without, mumps, so these have been combined. In Table 5 it is seen that sequelae were present in 10 of the 25 JBE cases, in 3 of 14 of the cases of ME, etc. Of the ten JBE cases who later were found to have residua, three had been in coma, and had had convulsions, and personality and reflex changes had been noted in all ten.

On reviewing this table, it is also noted that

TABLE 5

Acute illness characteristics and sequelae among survivors of 1947–1948 encephalitis epidemic on Guam*

Type of encephalitis	Sequelae		Number of patients who had the specified acute illness characteristics				
			Stupor	Convulsions	Coma	Personality changes	Reflex changes
Japanese B	Present	10	8	9	3	10	10
	Absent	15	9	3	3	8	12
	Total	25	17	12	6	18	22
Mumps	Present	3	0	0	0	0	2
	Absent	11	3	0	0	2	8
	Total	14	3	0	0	2	10
Unknown type	Present	2	2	0	0	2	1
	Absent	2	1	2	0	2	2
	Total	4	3	2	0	4	3
All types combined	Present	15	10	9	3	12	13
	Absent	28	13	5	3	12	22
	Total	43	23	14	6	24	35

* For definitions of classifications by these criteria see Edgren *et al.*, (1958).

among the JBE cases, 8 of 17 patients with stupor later developed sequelae, 9 of 12 who had convulsions later had sequelae, etc. It can be seen from this table that, among the JBE cases, most of those who had convulsions, stupor, coma, personality or reflex changes during the acute illness later developed sequelae.

Only a few patients with ME had severe symptoms during the acute illness and in these cases the symptoms are of little value for prognostication. For the "unknown type" there are too few cases to draw any conclusions regarding the prognostic value of the clinical symptoms and signs.

DISCUSSION

The clinical and serological studies which were carried out during the 1947–1948 encephalitis epidemic on Guam have afforded us an unusual opportunity for a type of prospective study which may clarify some unanswered and controversial points.

From the practical standpoint, it is desirable to know what outcome can be expected for various types of encephalitis. Our clinical experience with JBE has been almost totally dependent on two sources of data: epidemics among the Japanese described by Japanese scientists and the small series of cases among civilians and among U. S. Armed Forces personnel observed by U. S. Medical Officers stationed in the Far East.

The observations made during and following the JBE epidemic on Guam indicate that death or complete recovery are not the only alternatives, as many of the data, particularly from Japan, have suggested.

The case-fatality ratio in Japan was said to be 50 to 75 per cent in the epidemics of 1924 to 1937, and even as late as the Tokyo epidemic in 1955 (Mattison Comm. Rep't., 1939; Lewis, *et al.*, 1947), a mortality of 25 to 30 per cent was reported in the age group under 20 years. In the earlier Japanese outbreaks, most cases were said to occur among adults. In more recent epidemics, the proportion of children affected has apparently increased considerably but the case-fatality rate is still reported as high as 35 to 50 per cent (Baker, 1957; Tigertt, *et al.*, 1950). In 401 civilian cases observed in Okinawa from 1945 to 1949, the case-fatality rate was 35 per cent. During the same period a case-fatality rate of 28 per cent was noted among 21 cases in Americans (Tigertt *et al.*, 1950). These authors noted that "the severity of the disease and the fatality are intimately associated with the type of medical care and the general health of the individuals concerned." The high case-fatality rates "may also reflect incompleteness of reporting or finding of the milder cases" since the high fatality rates are generally from hospitalized cases.

It is probable that the high case fatality reported in Japan has been influenced by the attitude of "last resort" toward hospitalization by the Japanese patients in the past. Often only the most severely affected were hospitalized, and medical care frequently provided only the bare essentials. The increasing proportions of children may well represent a changing attitude in Japan toward hospitalization for sick children rather than any change in the age-specific infection rate.

According to Lewis *et al.*, (1947) and others (Kenelso and Aoki, 1928), sequelae are uncommon as reported by the Japanese; only 3 to 10 per cent are said to show residual effects. The low incidence of sequelae reported in Japan may be influenced by the inherent difficulties in providing a thorough follow-up examination. Often individuals who could not be found at a later date were eliminated, and the period after illness in which the later examinations were made was frequently too short to rule out permanent defects, particularly in infants and children.

A second source of data on JBE has been the experience of the U. S. Armed Forces stationed in the Far East (Lewis *et al.*, 1947; Tigertt *et al.*, 1950; Aring, 1949; Lincoln *et al.*, 1952; Weaver, 1957; Simpson *et al.*, 1947; Rivers, 1952). Among the young adult males in the U. S. Armed Forces, the case fatality in some of the large series of cases was about 11 per cent and sequelae were observed in 25 to 30 per cent of the survivors after 6 months. These sequelae included neuroses which may or may not have been related to the encephalitis (Aring, 1949; Weaver, 1957).

According to Lewis *et al.*, (1947) the case-fatality rate was 20 per cent among 66 Okinawan patients observed in a hospital manned by medical personnel of the U. S. Army and Navy in Okinawa; and in about one-fourth of the survivors "significant permanent disorders of the nervous system of variable degree" were noted. Of the 66 patients only two were over age 30 years, most were infants and children. In the same year and during the following 4 years, under the care of native physicians, the case fatality rate is said to have been about 35 per cent (Tigertt *et al.*, 1950).

At time of discharge from the hospital (frequently in a month or less after onset) about one-third of the Okinawan survivors observed by Lewis and associates had clinical evidence of mental impairment, personality changes, or disturbed motor function. Allowing for the possibility of further improvement, the authors estimated that one-fourth of the survivors will have suffered permanent damage of varying degree as a result of the disease. "Among the serious results to be anticipated, on the basis of information available from this outbreak and from the review of known postencephalitis, are hemiplegia, hemiparesis, aphasia, cerebellar syndromes, modified paralysis agitans, mental impairment, organic psychotic states, and idiocy associated with extreme deformity and paralysis" (Lewis *et al.*, 1947).

Simpson and Meikeljohn (1947) attempted a 1-year follow up of Okinawan civilian and American military patients who were hospitalized during the 1946 epidemic on Okinawa. Because of the confusion related to postwar relocation of the population it was possible to locate only 38 of the 77 civilian survivors. After a general physical examination, a neurological examination and a mental-status examination, 11 of the 38 (29%) were found to have sequelae; three of these 38 were severely incapacitated.

The authors felt that the incapacitated individuals were probably easier to locate in the follow-up and that the incidence of sequelae would have been lower if all the surviving patients had been examined, but in no case could it have been less than 14 per cent. However, this is still higher than that reported in the Japanese epidemics of 1924 and 1928 in which only 3.1 per cent of 2,000 affected are said to have sequelae (Kenelso and Aoki, 1928). However, the criteria used for the diagnosis and sequelae make difficult any comparison with the Japanese workers' reports. As Simpson and Meikeljohn (1947) point out, the low frequency of sequelae in the Japanese study is difficult to assess, since the reported case fatality rate of between 50 and 75 per cent in that series suggests that the more severely ill persons were hospitalized.

Recently, Weaver (1957) has discussed the results of extensive psychiatric studies on a group of American servicemen who had JBE. These men were studied during their acute illness and were followed at 3-months and 6-months intervals after recovery. During the acute illness anxiety was extremely common, arising primarily from a lack of correct information and a large variety of false ideas about the nature and after-effects of the disease. Of some 200 cases studied initially, complete follow-up interviews were obtained in over 100. Although a very few patients were discharged from the Armed Forces because of neurological sequelae, no evidence was found of any psychiatric sequelae in the absence of neurological sequelae.

The observation by Edgren *et al.*, (1958) that practically all survivors in the Guam epidemic observed 3 or 6 months after the acute episode were without sequelae is at variance with our present observations. It must be re-

called, however, that many of the patients were infants or young children at the time of the epidemic and that their intellectual and physical state was not so difficult to evaluate at this later date. Furthermore, we were fortunate in having available to us excellent bilingual interpreters, the village nurses, who were usually well acquainted with the capabilities and health status of each of the villagers.

In view of the frequent history of coma and convulsions in the patients and, in the deceased, the pathological findings of widespread lesions in the basal ganglia, anterior horn cells, cerebrum, and cerebellum as well as the thalamus, we were particularly interested in determining if survivors had seizures, mental retardation, cerebellar abnormalities, muscle atrophy, or any indication of basal ganglia defect. We also wished to determine if the long-term follow-up provided some indication that ME led to any severe neurological defect or if the combination of mumps and JBE gave rise to more sequelae than JBE alone. Still other aspects to be considered were the relation of the course of the acute illness to the development of sequelae, and any indication of what may predispose a person to develop clinical encephalitis when the large majority of the persons in the population have only inapparent infections.

In a retrospective study of patients with paralysis agitans observed on Guam a few years ago, a history of an acute febrile illness during the epidemic period was obtained in several (Mulder *et al.*, 1954) and it was assumed that such individuals suffered from JBE. However, none of these patients was included in the verified encephalitis cases reported here, and it remains to be seen whether JBE may be responsible for cases of Parkinsonism as the encephalitis patients reach the age of greater incidence of Parkinsonism.

No pure muscle atrophy such as would be found in poliomyelitis was noted among these patients. However, one girl, age 7 months at onset, had slight atrophy and weakness of the muscles of the left arm, but this was associated with a slight increase in tone, occasional convulsions, and right astereognosis as evidence of left cerebral damage.

Only one patient, a boy age 2 years at onset, had symptoms and signs of brain-stem dysfunction. When examined in March, 1957 he gave a history of intermittent diplopia, vertigo, and occasional "blank periods," and on examination had bilateral sucking reflexes, left lateral nystagmus and bilateral plantar extensor reflexes.

At the time of this study in four patients mental retardation appeared to be the consequence of JBE. The youngest survivor of the epidemic, age 6 months at onset, was severely retarded, and presented a picture of severe brain damage. The baby was crawilng when she became ill, but did not crawl again until age 1.5 years. At 10 years of age she could say only a few words of one or two syllables and played with 4- and 5-year-old children, primarily as a spectator. The other three children, ages 7 to 9 years at onset, were less severely retarded, but by comparison of their general information and their achievements in school, retention, recall, recent and past memory, it was obvious that these three were moderately retarded mentally and may have difficulty in making satisfactory socio-economic adjustments in adult life.

One patient, mentioned above, developed seizures subsequent to JBE. This girl had grand mal seizures with febrile illnesses almost yearly for 7 years. One other patient who was thought to be normal until 1953 developed seizures after being struck on the head with a rock. No fracture was seen on x-rays of the skull, but about 1 month later he began having daily grand mal seizures, which were not controlled until he was placed on anticonvulsants in 1957. Whether the encephalitis predisposed this patient to seizures is a matter of speculation.

Although the combination of mumps and JBE would seem more severe than JBE alone, no difference as to frequency or severity of sequelae, has been noted between the two groups of patients.

Of the 11 patients with ME who were followed, none was aware of any defect although three were found to have signs of neurological deficit, such as a Babinski sign or mild dysdiadochokinesia. All three were gainfully employed full time. All three were young adults at the onset of illness. Although it is possible that they might have had mild cases of JBE, the results of the serological studies make this very unlikely No evidence of intellectual or psychiatric impairment was found in any of the mumps patients who were followed.

Very little can be stated about sequelae of the

cases of encephalitis, type unknown, which occurred during the same period as the mumps and JBE. Apparently it was a relatively severe form of encephalitis since all the survivors had neurological deficits at the time of this study. The one death in this group, a 4-year-old child, probably was due to JBE although serological studies were inconclusive. Two other cases, men ages 26 and 57 years at onset, might have had JBE, as the complement-fixation titer against JBE rose, whereas the neutralization titer did not rise. If any or even all the cases of encephalitis, type unknown, were caused by the virus of JBE, the frequency of sequelae would be still greater for the Japanese B group than that which we reported earlier in this paper.

Three of the 43 survivors had pre-existing neurological abnormalities; two were considered mentally retarded prior to the encephalitis and one young lady had evidence of Charcot-Marie-Tooth disease (Mulder *et al.*, 1954). Although no definite statement can be made, one wonders whether pre-existing central-nervous disease might be a factor that predisposes the few among the many who are infected with virus to clinical encephalitis.

SUMMARY AND CONCLUSIONS

During the mumps and Japanese B encephalitis (JBE) epidemics of 1947–1948 there were 46 known cases of encephalitis among the Guamanian civilian population, with 43 survivors of the acute illness, giving a case fatality of 6.5 per cent overall. Two deaths occurred among the 27 patients diagnosed as having JBE (7.4%). Follow-up examinations were made more than 9 years after the acute illness on all 32 of the survivors who still live in Guam and in six of the 11 other patients who have moved elsewhere.

Twenty-three of 25 survivors of JBE, 11 of 14 survivors of mumps encephalitis (ME), and all 4 survivors of an unknown type of encephalitis were examined. All survivors not examined were known to be living at the time of this study and on the basis of available information are presumed to be well. The frequency of neurological or psychiatric sequelae in all survivors at the time of follow-up examination was at least 40 per cent (11 per cent severe; 29 per cent mild) for JBE; 22 per cent (all mild) for ME; all survivors of the unknown type of encephalitis had neurological abnormality at the time of follow-up. The sequelae noted in the survivors of JBE, in decreasing frequency, were: pyramidal-system involvement, mental retardation, impairment of cortical sensory association, psychiatric complaints, brain-stem syndrome, cranial-nerve lesion, muscle atrophy and convulsions.

Only three patients with ME had sequelae and these were very mild. Two had bilateral Babinski reflexes, which suggests that ME may be one reason for the occasional presence of this pathological reflex in otherwise normal people.

Sequelae were much more common in the patients who were less than 10 years of age at time of onset of JBE. Infants had more severe sequelae than older children.

The presence of convulsions and other signs of neuronal involvement during the acute illness correlated well with the later presence of sequelae of JBE. Other symptoms and signs such as elevated temperature, vomiting, and spinal-fluid changes were not of particular prognostic significance.

The high case fatality (33% or higher) and the low sequelae rates reported in the Japanese studies (3 to 14%) contrast with the report of encephalitis cases in this study. The excellent medical care available to the population of Guam may account in part for the low mortality and the high incidence of sequelae reported here. This care may have been responsible for the survival of many patients with severe damage to the brain who would have succumbed under other conditions of hospitalization.

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POTENTIAL FOR BIOLOGICAL CONTROL OF *AUSTRALORBIS GLABRATUS*, THE INTERMEDIATE HOST OF PUERTO RICAN SCHISTOSOMIASIS

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INTRODUCTION

Experiments for control of schistosomiasis in Puerto Rico are based presently on chemical destruction of the only snail host, *Australorbis glabratus*, improvement in soil and stream sanitation by privy installations, proper design and operation of sewage treatment plants, improved domestic water supplies, health education, and limited chemotherapy. The first of these seems to be the most practical but some recent reports indicate that biological control should be investigated. Oliver-Gonzalez *et al.*, (1950, 1956) reported that *Australorbis glabratus* colonies in a stream and pond had been displaced by *Marisa cornuarietis* which had been introduced into the habitats. Chernin, *et al.*, (1956, 1956a) studied the details of the impact of the two species in laboratory colonies. The present paper gives the results obtained in extensive field observations after the introduction of *Marisa* into new habitats. Studies on the biology of this species have been published elsewhere (Ferguson and Palmer, 1958). Observations were also made on three other species of snails, *Helisoma duryi eudiscus*, *Ampullaria* sp. and *Tarebia granifera mauensis*, that have been introduced into Puerto Rico in recent years.

FIELD TESTS OF *AUSTRALORBIS* CONTROL BY *MARISA*

The habitats into which *Marisa* was introduced included 16 farm ponds, the watershed of the Quebrada San Anton, and six streams on Vieques Island. *Australorbis* was present in eight of the ponds and the other eight were used to determine whether *Marisa* could become established and prevent colonization of the vector species. Most of the colonies were started with a single manual planting of at least 200 snails from laboratory tanks. Observations on these ponds began in 1955 and were made either monthly or quarterly. Under optimum conditions a period varying from about 6 months to 1 year was required to reach the population-carrying capacity of a pond, and at that time *Marisa* declined to a static level according to the amount of food available. In five of the ponds, *Marisa* established flourishing colonies in less than a year. The snail developed more slowly in five other ponds, was grown with difficulty in five more, and a colony could not be established in one pond, probably because it was new and therefore lacked food. Eight of the ten ponds in which *Marisa* colonies were easily established originally contained *Australorbis*. The latter snail disappeared from all eight of these ponds by November 1957, in an average time of 8 months.

During March 1956, the entire watershed of the Quebrada San Anton, about 12 square miles, was surveyed for *Australorbis* colonies in the main stream and its 26 tributaries, various paralotic swamps, and oxbows. *Australorbis* colonies ranging in density from 0–1 to 5–20 per square yard were plotted on a detailed map, and *Marisa* was transplanted to these habitats, usually in batches numbering more than 200 snails. Distribution downstream in the main stream and larger tributaries from plantings at stream origins was aided by storm action. Such action killed many *Marisa*, but also cast them into new foci. Two years later it had not become well established in certain high-velocity sections of the main stream, or in one place some 500 yards downstream from a gross source of pollution. Large-scale housing developments and channel rectifications have destroyed some plantings. However, the *Marisa* continue to expand their distribution in the watershed, surviving storms in which *Australorbis* die. *Australorbis* definitely has become less abundant

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in the study area since 1956, now being absent from almost all of the streams, as judged from quarterly surveys extending through June, 1957. At that time only four small colonies of *Australorbis*, of less than one snail per square yard, could be found in the watershed. None was infected. The September, 1957 survey revealed *Australorbis* localized in only three very sparse colonies.

Each survey reveals likely places for *Marisa* to flourish, and plantings are made in such locations, usually averaging about six placements. The principal food consists of *malanga*, an "elephant ear" type of aquatic plant which is abundant.

Massive colonization of *Marisa* in this watershed appears definitely to be responsible for gradual disappearance of *Australorbis*. Studies of the long-term effect of this type of control on the schistosomiasis index among watershed residents are currently under way.

Marisa now is being used on an experimental basis as an adjunct to other types of *Australorbis* control by the Bilharzia Control Unit, Department of Health, Commonwealth of Puerto Rico. For example, as a preventive measure, *Marisa* were planted in Vieques Island and Mariana Basin streams which had been treated previously with sodium pentachlorophenate for *Australorbis* control. In six Vieques streams where this procedure has been used, *Australorbis* has not returned 12 months following eradication.

AUSTRALORBIS CONTROL BY OTHER SNAILS

The planorbid *Helisoma duryi eudiscus* gained attention because of the great population densities noted in one pond in the absence of *Australorbis*. It was planted in three well-balanced farm ponds or irrigation-water storage tanks well supplied with *Najas*, and has resulted in the disappearance of average-size colonies of *Australorbis*. *Helisoma* is usually buried in mats of vegetation in depths up to 3 feet. It has not become established in one farm pond containing matted *Chara* after four attempts, and the development in one irrigation tank has been slow. *Helisoma* growth in two tanks was explosive accompanying concomitant decline and disappearance of the *Australorbis* colonies in about 6 months. Two attempts during October, 1955 at starting *Helisoma* colonies in small streams were abortive, yet it has appeared spontaneously during 1956 at a well-known station in the Rio Caguitas below an Imhoff tank outfall, and has thrived to the decided limitation of *Australorbis* normally abundant there. In St. Croix, V. I., *Helisoma* was planted in four ponds by pet-shop owners in 1954 and became the dominant species in less than 1 year in the absence of *Australorbis*. *Helisoma* is able to survive for 4 months after drying and mechanical cleaning of ponds. The mechanism of the competition between *Helisoma* and *Australorbis* should be studied.

The large, globose, ampullarid *Ampullaria* sp., or apple snail, was planted in the Rio Caguitas in 1954 by a tropical-fish fancier (Watlington, 1955) and has since spread up and down this and an adjoining stream in great profusion. In limited areas, principally in quiet or polluted sections, it thrives if abundant surface material is present, such as concrete walls or wood, on which to lay its eggs. It will not establish itself in ponds with gentle slopes and without egg-attachment surfaces. It is under field study in three ponds, two swamps, three rivers, one creek, and one lake, but *Ampullaria* has shown no promise to date as a significant competitor of *Australorbis* in these studies.

The snail, *Tarebia granifera mauensis* (family Thiaridae), a known host of human paragonimiasis in Asia, is also being observed. It was introduced into the island from unknown sources presumably during 1956. Although it is small, developing a cone 25 mm in length and 8 mm at greatest diameter, it produces unbelievably large colonies in short periods, by the process of parthenogenesis, which blanket the marginal areas of streams and impoundments. There is growing evidence in the Rio Caguitas that *Tarebia* interferes with *Australorbis*, based on marked increases of the thiarid and definite decreases in numbers of *Australorbis* checked regularly at five stations on a monthly schedule. During September, 1957, *Australorbis* could be found at only two of the five stations, while *Tarebia* could be collected at the rate of over 200 per dip. *Tarebia* may also compete in ponds and lakes, since the only large lake in Puerto Rico which during 1956, did not contain *Australorbis* was crowded with *Tarebia*. This species is being widely and rapidly spread in central watersheds, probably by attachment of its rough shell to plant materials and animals.

Predation and competition among species of snails are therefore, judged most promising in inhibiting the colonial growth of *Australorbis*.

For more information on biological control of mollusks refer to the excellent review of Michelson (1957).

SUMMARY

Field trials are demonstrating the valuable suppressive effect of interspecific competition by *Marisa cornuarietis* on *Australorbis glabratus*, the snail intermediate host of Puerto Rican schistosomiasis. *Marisa* colonies were established easily in ten of sixteen farm ponds planted manually with the snail. Eight of the ten ponds originally contained *Australorbis*. By about 2 years after planting, *Australorbis* had disappeared from all eight of the ponds, in an average time of 8 months. Widespread planting of *Marisa* in one endemic watershed has practically eliminated the schistosome host there in less than 2 years. *Marisa* is now being used as an adjunct to other types of *Australorbis* control by the insular government.

Among other snails under study *Helisoma duryi eudiscus* shows definite promise of useful competition in irrigation tanks. *Tarebia granifera manuensis* appears to be effectively crowding *A. glabratus* in some areas, while *Ampullaria* sp. has been ineffective.

Other than snails, there apparently are few natural enemies of *Australorbis* in Puerto Rico.

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STUDIES ON THE HOST PARASITE RELATIONSHIPS TO *SCHISTOSOMA JAPONICUM*

I. The Effect of Single Graded Infections and the Route of Migration of Schistosomula[1]

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A review of the literature reveals presumptive evidence that resistance to schistosomiasis occurs in man and animals. However, very little information is available as to the degree of this resistance, the time at which it develops, and the nature and functioning of the mechanisms involved. Also, much still needs to be known about the pathogenicity and the route of migration of the parasites within the mammalian host.

The present series of investigations was conducted in an attempt to fill some of the gaps remaining in the knowledge of the relationship between *Schistosoma japonicum* and its host. The effect of single infections of varied doses on the survival of mice and on the per cent of worms recovered was investigated. Furthermore, experiments were carried out to determine whether schistosomula reach their definitive site in the portal vessels through the circulatory system or through tissue migration.

MATERIALS AND METHODS

Young male adult and suckling white mice were used in the experiments. They were segregated, five to a cage, and fed mouse pellets, occasionally supplemented by cottage cheese. Some mice were exposed to between 20 and 300 cercariae each, and others served as uninfected controls. The day before exposure to infection all of the animals were shaved on their flanks and backs.

Cercariae of *S. japonicum* were obtained by crushing infected snails collected from endemic areas. Not less than ten infected snails were crushed at one time to assure the presence of both sexes among the infecting cercariae. The cercariae were counted and the animals infected as described elsewhere (Sadun and Walton, 1958). Following exposure, the animals were removed to their cages and the skin permitted to air-dry.

All of the mice used for the studies of single graded infections were kept for 9 weeks before being sacrificed. Necropsy was performed on animals that died before that time to determine whether death might have been produced by adventitious causes. In each case the perfusion technique (Pan and Hunter, 1951) was used for recovery of the developing schistosomes.

The mice used to study the route of migration of schistosomula were sacrificed at intervals of 24 hours. The thoracic and abdominal cavities of adult mice were opened separately, carefully avoiding copious bleeding, and rinsed with normal saline. The fluids were then collected, centrifuged and the sediment examined under the microscope for recovery of schistosomula which might have been present. The lungs, heart, diaphragm and liver were removed, fixed in 10% formalin, embedded in paraffin and sectioned. When suckling mice were used the heads and appendages were removed, and the body was fixed *in toto*. Sections were cut 7 μ thick, mounted serially, and stained with Delafield's hematoxylin and counterstained with eosin. By this method the parasites were well stained and could easily be differentiated from the surrounding host tissues.

RESULTS

1. *Lethal infections.* Three series of experiments were carried out to study the effect of graded infections on the survival of mice. In each series mice were divided into eight groups and exposed to varied numbers of cercariae from 0 to 140. In view of the similarity of the results obtained, the three series were combined and the results were summarized in Table 1.

All together 108 animals of 194 died as a result of the infection with *S. japonicum*. Six other mice died of extraneous causes (pneumonia, cannibalism), and are not included in these data. Deaths of the experimental animals occurred between the twenty-first and the sixtieth day after inoculation, with an average of 44.5 days.

[1] With the technical assistance of Mr. Yoshimasa Hishinuma.

TABLE 1

Effect of graded single exposures to Schistosoma japonicum *cercariae on survival of mice and recovery of worms*

Group	Mice	Cercariae	Results within 9 weeks from time of inoculation								
			Dead	Accum. total % mortality	Mean time from inoc. to death	Worms recovered in dead mice		Worms recovered in survivors		Worms recovered in group	
						% of inoc.	Ratio ♂/♀	% of inoc. ♂/♀	Ratio ♂/♀	% of inoc.	Ratio ♂/♀
I	20	0	0	0	—	—	—	—	—	—	—
II	24	20	4	6	56	82	1.1	30	1.5	40	1.4
III	24	40	11	25	52	60	1.5	22	2.0	41	1.4
IV	29	60	21	52	50	56	1.3	22	2.1	45	1.4
V	24	80	15	67	47	54	1.4	30	1.5	45	1.5
VI	26	100	21	82	46	56	1.7	17	9.6	45	1.9
VII	28	120	24	90	38	61	1.4	5	2.0	53	1.4
VIII	19	140	12	94	37	52	1.7	22	0.7	43	1.5

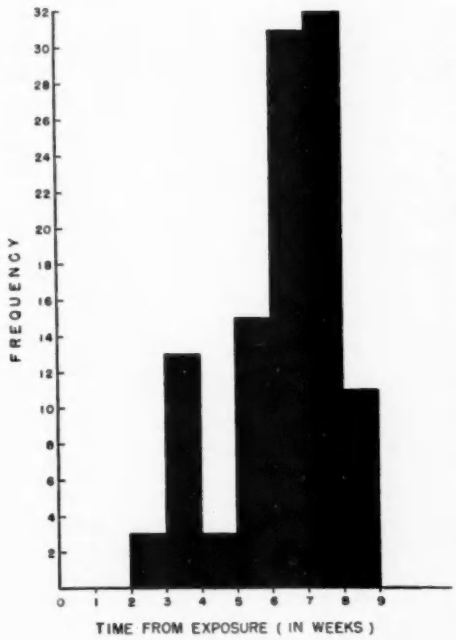

FIG. 1. Frequency distribution of time of death in mice exposed to graded doses of *Schistosoma japonicum* cercariae.

The greatest number of mice, however, died between 7 and 8 weeks following infection (Fig. 1). The frequency distribution in time from inoculation to death showed two distinct periods during which mortality occurred, with peaks between 3 and 4 weeks and 6 and 8 weeks following inoculation. This frequency distribution of death is in agreement with necropsy observations and with the life cycle of the parasite. Most of the animals which died before the fifth week were in the groups exposed to very large doses of cercariae. The apparent cause of death was due to blockage of blood vessels by large numbers of young adults. Most of the mice that died after the sixth week from exposure to infection came from the groups which received somewhat milder doses. At necropsy no blocking of blood vessels was seen in these animals but the liver and intestine showed the presence of large numbers of parasite eggs. Attention is called to the fact that oviposition takes place just prior to this time.

As indicated in Table 1, in general the size of the inoculum was found to have an inverse relation to the average time between exposure to infection and death. While not always significant from one size of inoculum to another, the difference between average survival times of mice exposed to 20 cercariae (Group II) and mice exposed to 140 cercariae (Group VIII) is highly significant. The LD_{50} was estimated by the method of Reed and Muench (1938) to be 58 cercariae.

2. *Relationship between size of exposure and development of worms.* In general, the increase in numbers of cercarie to which mice were exposed brought about an increase in the mean number of worms recovered. The per cent development of

TABLE 2

Numerical distribution of Schistoma japonicum *in adult mice on successive days after exposure*

Numbers of cercariae		300	100	300	100	300	100	300	300	300	300	100
Days after infection		1		2		3		4			5	
Lungs	alveoli	0	0	1	5	26	20	43	NS*	NS	4	7
	alveolar wall	0	2	3	3	2	3	4	NS	NS	10	5
	capillaries	0	0	2	0	1	0	2	NS	NS	0	1
	bronchi	0	0	0	0	0	0	0	NS	NS	0	0
	Total	0	2	6	8	29	23	49	NS	NS	14	13
Diaphragm		0	0	0	0	0	0	1	0	0	0	0
Liver	parenchyma	0	0	0	0	0	0	3	0	0	0	0
	central veins	0	0	0	0	1	0	2	23	20	4	20
	portal veins	0	0	0	0	1	0	1	5	13	13	7
	Total	0	0	0	0	2	0	6	28	33	17	29
Pleural cavity		0	0	3	0	1	8	0	0	0	11	3
Peritoneal cavity		2	0	3	0	0	0	0	0	0	0	0
Total per mouse		2	2	12	8	32	31	57	28	33	42	45

* NS = No sections studied.

TABLE 3

Numerical distribution of Schistosoma japonicum *in suckling mice on successive days after exposure*

Number of cercariae Days after infection		50 1	50 2	200 3	200 4	200 5	200 6	50 7
Skin	epidermis and corium	9	0	0	0	0	0	0
	subcutaneous	38	3	12	0	0	0	0
	Total	47	3	12	0	0	0	0
Lungs	alveoli	0	3	1	0	0	0	0
	alveolar wall	0	5	65	0	1	0	0
	capillaries	0	2	4	0	0	0	0
	Total	0	10	70	0	1	0	0
Diaphragm		0	0	0	0	0	0	0
Liver	parenchyma	0	0	4	7	4	50	1
	capillaries	0	0	1	10	16	51	1
	portal veins	0	0	4	11	8	65	3
	Total	0	0	9	28	28	166	5
Mediastinum		0	0	1	0	0	0	0
Heart		0	0	1	0	0	0	0
Pleural cavity		1	0	2	1	0	0	0
Total per mouse		48	13	95	29	31	166	7

adults was similar in all groups regardless of the number of cercariae to which the animals were exposed.

More male schistosomes than females were recovered in each group. The ratio varied from 1.4 (Groups II, III, IV, and VII) to 1.9 (Group VI) with an overall average of 1.5. The sex ratio of over 2,000 worms recovered in previous experiments conducted in this laboratory was 1.3 males per female. A significantly greater per cent development of adult schistosomes occurred in each group among the mice that died as a result of infection, than among those which survived with a single infection. Whereas the ratio between male and female worms among the mice that died did not deviate greatly (from 1.1 in Group II to 1.7 in Groups VI and VII) the opposite was true among the mice which survived the infection (from 0.7, Group VIII to 9.6, Group VI).

The per cent development of worms observed in the present experiments compares favorably with that observed previously. In the course of 21 experiments carried out in this laboratory in the past, involving a total of 233 mice which were exposed to between 10 and 100 cercariae each, 55 per cent of the inoculum was recovered at necropsy.

3. *The route of migration of parasites.* In the first experiment 11 adult mice were selected. Seven mice received 300 cercariae each and four 100 cercariae each. Two mice were sacrificed 1, 2, 3 and 5 days after exposure to infection. Three mice were sacrificed 4 days after infection. The results of examination of pleural and peritoneal washings and of serial sections are summarized in Table 2. Only two schistosomula were found in the lungs one day after infection. The number increased progressively 2, 3 and 4 days after infection and began to decrease on the fifth day. In general, the young worms were about equally divided between the alveoli and the alveolar walls. No schistosomula were found in the liver of mice sacrificed during the first 2 days following infection. Only two were found 3 days following infection. Four and 5 days after infection they were present in relatively large numbers. The majority of young worms found in the liver were in the central veins. Many worms, however, were observed in the portal veins and a few were seen in the parenchyma immediately beneath the anterior surface of the liver. In one instance a young worm was observed in the diaphragm.

As indicated in Table 2, only a relatively small number of young worms was recovered from the mice. The relatively small rate of recovery of worms had to be attributed to the fact that only a small portion of the lungs, diaphragm and liver could be sectioned and studied so that a considerable proportion of the young worms residing in organs other than those sectioned was missed.

To obviate this source of possible error of interpretation, a second experiment was carried out with suckling mice so that the entire animal, with the exception of its head and appendages, could be fixed *in toto*, serially sectioned, stained and studied under the microscope. A series of seven mice was used for this experiment; three were exposed to 50 cercariae each, and four to 200 cercariae each. One animal was sacrificed each day for 7 days following infection. The results are summarized in Table 3.

Since, in these young animals, one could not always differentiate with certainty between capillaries of the efferent and afferent vessels in the liver, the term capillaries was used in the table to include both. One day after infection all the schistosomula recovered were in the skin except for one in the pleural and one in the peritoneal cavities. Most of the cercariae in the skin had already reached the subcutaneous tissue. Two days after infection most of the schistosomula had already migrated to the lungs. Three days after infection some schistosomula were still present in the skin but most were in the lungs where, for the most part, they could be found in the alveolar walls. At this time some young worms had reached the liver where they were almost equally divided between the parenchyma at the anterior face of the liver and the portal vein. One young worm was found in the heart muscle, one in the mediastinal tissue and two in the pleural cavity.

Beginning with 4 days after infection, all but one of the schistosomula recovered were in the liver. Essentially the same worm distribution was observed 4, 5, 6 and 7 days following infection. At this time the young worms were nearly equally distributed between the parenchyma, the capillaries and the portal veins.

Attention is called to the fact that by sectioning suckling mice *in toto* 389 young worms were recovered out of a total 950 cercariae to which the mice had been exposed. This figure is not appreciably different from the per cent of recovery obtained in the previous series of experiments

on the effect of graded single exposures, thus indicating that few, if any, schistosomula have been overlooked in examining serial sections of entire suckling mice, or were present in the discarded head and limbs.

DISCUSSION

The results of single graded doses of *S. japonicum* cercariae in mice indicate that beyond a certain threshold the infection causes their death. Death was due primarily to blockage of the blood vessels in very heavy infections and to the effects of oviposition in lighter infections. As was to have been anticipated from previous data, the size of infection had a direct relation to the per cent mortality in each group and an inverse relation to the average time between exposure to infection and death. The per cent development of adults did not vary significantly with different sizes of infecting doses.

Important factors bringing about the death of mice in each group were the total number of worms developing and the presence of a corresponding number of male and female schistosomes within each animal. In general, it appeared that whenever there was a great difference between the number of male and female worms present in the host, no deaths occurred even when the total number of worms was relatively high.

The results of these experiments also indicated that, as a rule, more male than female parasites were recovered when animals were infected by pooling the cercariae from a large number of snails. The ratio between sexes in the infecting cercariae appeared to be consistent.

It has been well established by previous investigators that the cercariae of *S. japonicum* enter the mammalian host primarily through the skin, gain access to the lymph and blood vessels and reach the lungs through the heart and the pulmonary arteries. Olivier (1952) found that *S. japonicum* in mice reach the portal system quickly after a rapid passage through the lungs. No general agreement, however, exists as to the route of migration from the lungs to the liver. Narabayashi (1915, 1916) and Sueyasu (1920) believe that the young worms leave the lungs by the way of the pleural cavity, penetrate the mediastinal tissues or the diaphragm and then penetrate the liver, the portal and the mesenteric veins. On the other hand Faust and Meleney (1924) concluded from their observations that the main course taken by the young *S. japonicum* worms from the lungs to the liver is by way of the pulmonary veins, the arterial circulation, the gastrointestinal capillaries, and the mesenteric and portal veins. The observations made in the course of our experiments during which over 15,000 sections were studied, do not agree entirely with either of these opposing views.

In agreement with Faust and Meleney (1924), in our experiments most of the worms appeared to migrate from the lungs to the liver through the blood vessels. This was evidenced by the following observations: 1) The young parasites reached the liver very rapidly, ordinarily within 3 to 4 days whereas migration through tissues would be expected to take a longer time. 2) If migration through tissues had occurred to any great extent more schistosomula should have been observed in the pleural and peritoneal cavities, whereas only small numbers were found. 3) Even assuming that schistosomula in these cavities had been overlooked in the course of our observations, evidence of mechanical damage would have been observed in the diaphragm and in the liver, whereas, no signs of such damage were observed.

On the other hand, contrary to Faust and Meleney's observations, in our experiments the young worms in the liver were not generally found in the portal veins. Approximately only one-third of the schistosomula found in the liver of adult mice, and two-thirds of those in the liver of suckling mice, were in the portal veins. These observations suggest that: 1) Some young worms reach the liver through the blood circulation, whereas others penetrate the parenchyma through the anterior surface of the liver. 2) Most of the worms reach the liver through the blood circulation, but after reaching the portal veins they migrate through the parenchyma and/or through the portal-hepatic venous anastomoses, to the central veins and back. No explanation is given for the absence of visible mechanical damage of liver tissue.

SUMMARY

Several groups of mice were infected with graded doses of *Schistosoma japonicum*. The 50 per cent lethal dose under the experimental conditions was 58 cercariae. The total number and the sex distribution of developing worms were important factors in bringing about death. In very heavy infections death was due primarily to thrombosis resulting from large masses of worms

and, in relatively lighter infections, to extensive oviposition. Study of over 15,000 serial sections from infected mice sacrificed at regular intervals indicates that most of the developing worms migrate from lungs to liver through blood vessels. However, only a portion of schistosomula found in the liver were in the portal veins. Large numbers of young worms were observed in central veins and parenchyma.

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STUDIES ON THE HOST PARASITE RELATIONSHIPS TO *SCHISTOSOMA JAPONICUM*

II. Quantitative Changes in the Concentration of Serum Proteins in Humans and Rabbits

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INTRODUCTION

Paper electrophoresis provides an ideal tool for understanding some of the mechanisms of pathogenesis of infectious agents and of the host response to them. In addition, in some diseases, at least, it provides pictures so distinctive as to serve as an aid in medical diagnosis. The application of electrophoretic techniques in the field of parasitic diseases and its limitations have been reviewed by Stauber (1954). Serum protein changes occurring in mice following *Schistosoma mansoni* infection have been described recently (Evans, *et al.*, 1957). Electrophoretic patterns of ten sera from *S. mansoni* patients with hepatosplenomegaly were previously reported by Fiorillo (1954).

The present study was set up to determine changes occurring in the relative proportions of serum proteins in rabbits at regular intervals following single infections with *S. japonicum*. Sera from human patients reporting to clinics with chronic and subacute infections with *S. japonicum* were also tested and compared with sera from normal individuals.

MATERIALS AND METHODS

Young adult male rabbits of approximately the same age and weight were placed in individual cages and fed a standard diet. They were bled from the marginal ear vein at regular intervals starting 1 month before exposure to infection. Stools were examined individually 1 month following exposure to cercariae and again thereafter to determine the presence of eggs. Some animals were exposed to between 250 and 1,000 schistosome cercariae each. Others were kept as uninfected controls. Before exposure to infection the rabbits were clipped and shaved over a large portion of the abdomen. Cercariae of *S. japonicum* were obtained by crushing infected snails collected from endemic areas. Large numbers of snails were crushed at one time to insure the presence of both sexes among the infecting cercariae. The cercariae were transferred with a wire loop to the center of a watch glass and counted directly under a dissecting microscope. After the desired number of cercariae was counted on each watch glass, each rabbit was held firmly with the shaved skin in contact with the water surface. A few minutes later the watch glass was examined again under the microscope. If cercariae were seen the procedure was repeated until all had attached themselves to the skin of the rabbit. Six months after infection rabbits were sacrificed by exsanguination under light anesthesia; the worms were recovered by perfusion, and counted.

A total of 63 sera from human beings were divided into three groups. One group included sera from proven cases of *S. japonicum* infection as determined by clinical and laboratory findings. Another group included sera from Japanese people living in nonendemic areas, who had never been exposed to infection with *S. japonicum*. A large percentage of these sera came from individuals harboring other intestinal parasites, primarily nematodes. A third group included sera from Americans who had never been exposed to infection with *S. japonicum*.

Total-serum-protein determinations were made by the biuret colorimetric technique. Fractionations were carried out in a semiclosed horizontal cell, using filter paper strips 120 mm long, and veronal buffer pH 8.6, ionic strength 0.07, for 4 hours at room temperature. A constant-voltage DC-power supply of 200 volts, 7.5 ma, was used. After fractionation, the strips were dried and stained with bromphenol blue, three rinses in 5% HAc and a final rinse in a 10% HAc, 2% NaAc solution. The slips were scanned, ionograms plotted and the areas under the density curves integrated.

RESULTS

1. *Serum patterns in infected rabbits.* A total of 134 sera from schistosome-infected rabbits and uninfected controls were collected at regular intervals for a period of 7 months. Five fractions were regularly identified in order of decreasing mobility. They are referred to as albumin, alpha-1 globulin, alpha-2 globulin, beta globulin and gamma globulin. The results are summarized and presented graphically in Table 1 and Figure 1. No marked alterations in the amounts of total protein were observed during the first 30 days following infection. Beginning with the forty-fifth day, however, a marked increase in total protein was evident among the infected rabbits. This increase continued to be demonstrable throughout the duration of the experiment.

The albumin fraction remained essentially unchanged the first month of infection. By the forty-fifth day, however, a sharp differentiation between the control and experimental ionograms occurred due to a marked decrease of the relative amounts of albumin in the infected rabbits. This decrease remained essentially unchanged for the rest of the experiment.

No significant constant differences between

TABLE 1

Effect of infection with Schistosoma japonicum *on concentration of serum proteins in rabbits*

Date from exposure in days	Experimental groups	No. of sera	Mean concentration of components (per cent)					A/G ratio	Total protein
			Alb.	$Alpha_1$	$Alpha_2$	Beta	Gamma		
−30	Noninfected	12	55.9	10.0	7.7	13.3	12.4	1.3	5.3
0	Noninfected	12	58.2	8.9	6.4	13.1	13.4	1.4	6.0
15	Noninfected	8	60.0	10.2	6.7	13.4	9.7	1.5	5.6
15	Infected	10	62.8	7.5	5.8	12.8	11.0	1.7	6.7
30	Noninfected	3	57.4	8.2	7.9	11.9	14.2	1.3	5.6
30	Infected	9	57.3	9.5	5.5	13.4	13.1	1.4	5.4
45	Noninfected	3	61.4	7.9	6.8	11.5	12.4	1.6	5.1
45	Infected	8	32.4	10.3	7.9	18.7	30.7	0.5	7.1
60	Noninfected	3	58.9	9.1	7.9	11.8	12.3	1.4	5.3
60	Infected	6	29.0	10.6	7.3	17.1	35.9	0.4	7.4
75	Noninfected	3	55.5	9.3	6.6	13.4	15.1	1.2	6.0
75	Infected	7	27.4	7.6	4.3	13.4	47.3	0.4	9.0
90	Noninfected	3	59.6	6.7	6.5	14.6	12.6	1.5	5.4
90	Infected	7	29.7	5.8	3.7	13.5	47.2	0.4	7.9
105	Noninfected	3	57.4	6.0	10.1	13.4	13.1	1.3	5.3
105	Infected	7	31.2	6.1	4.5	12.4	45.7	0.4	7.7
120	Noninfected	3	53.6	7.3	7.3	16.3	13.5	1.2	5.9
120	Infected	7	31.4	7.2	3.6	12.8	46.2	0.5	8.9
150	Noninfected	3	58.1	6.7	8.8	14.1	12.3	1.4	6.3
150	Infected	7	28.5	7.7	4.3	14.0	45.5	0.4	9.7
180	Noninfected	3	59.4	8.2	8.4	13.1	10.9	1.5	5.5
180	Infected	6	26.7	8.7	8.3	15.4	40.9	0.4	8.0

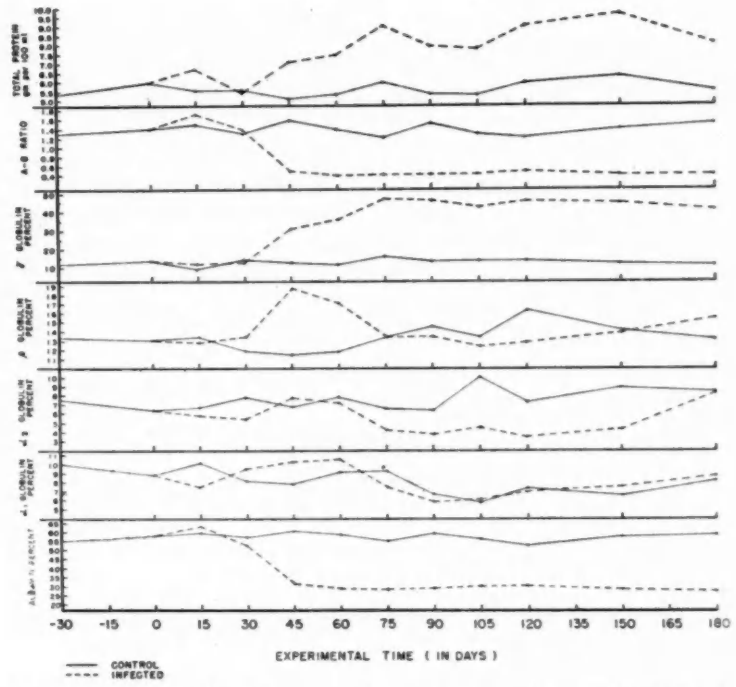
TOTAL PROTEIN gm per 100 ml
A-G RATIO
γ GLOBULIN PERCENT
β GLOBULIN PERCENT
α2 GLOBULIN PERCENT
α1 GLOBULIN PERCENT
ALBUMIN PERCENT
-30 -15 0 15 30 45 60 75 90 105 120 135 150 165 180
EXPERIMENTAL TIME (IN DAYS)
CONTROL
INFECTED

FIG. 1. Quantitative changes in the concentration of serum proteins in rabbits following infection with *Schistosoma japonicum*.

TABLE 2

Effect of infection with Schistosoma japonicum *on the concentration of serum proteins in humans*

Serum source	Number of sera	Mean concentration of components (per cent)*					A/G ratio†	Total protein
		Alb.	$Alpha_1$	$Alpha_2$	Beta	Gamma		
Infected Japanese	39	40.4 (±1.04)	8.7 (±0.5)	10.4 (±0.4)	14.7 (±0.5)	25.8 (±1.23)	0.68 (±0.04)	7.1 (±0.18)
Noninfected Japanese	14	46.7 (±0.76)	6.5 (±0.5)	10.6 (±0.4)	13.5 (±0.7)	22.7 (±0.7)	0.88 (±0.06)	6.6 (±0.22)
Noninfected Americans	10	46.7 (±1.18)	7.0 (±0.4)	11.8 (±0.6)	13.3 (±0.8)	21.1 (±1.38)	0.88 (±0.04)	6.7 (±0.28)

* Figures between parentheses indicate standard error.

† Data uncorrected for loss of albumin in migration.

the uninfected and the infected rabbits were observed in the proportion of alpha-1 globulin throughout the experiment. On the other hand, a decrease in the relative amount of alpha-2 fraction was noticeable after the second month of infection. No constant significant changes were observed in the relative amounts of beta fraction.

The most dramatic change occurring as a result

of infection was a sudden and marked increase in the gamma-globulin fraction, beginning with the forty-fifth day after cercarial penetration and continuing to the end of the experiment. The change in the total area included within the albumin and gamma-globulin regions was reflected in a striking decrease in the A/G ratio.

2. *Serum patterns in human patients.* The effects of infection with *S. japonicum* on the relative proportions of serum proteins are shown in Table 2. No significant changes in the relative proportions of alpha-2 and beta fractions were observed in the three groups. Also, no changes were observed in any of the serum fractions as between noninfected Japanese and Americans. On the other hand, there was a significant decrease in the amount of albumin and a significant increase in the total proteins and in the relative amounts of alpha-1 and gamma globulins in the infected Japanese as compared to the uninfected controls. This resulted in a significant reduction in the A/G ratio.

DISCUSSION

All the rabbits used in this study were of the same strain, age, weight and sex and were maintained under similar conditions with respect to diet and environment. This consideration, added to the fact that preinfection and postinfection controls were maintained throughout the experiment, indicates that the changes in the serum proteins observed in the experimental rabbits can be ascribed to schistosomiasis. The most marked changes resulting from infection were: the increase of total protein, the increase of gamma globulin and the decrease of albumin. All of these changes occurred between 30 and 45 days following exposure to infection. The overall picture remained essentially unchanged up to 6 months following infection. No tests were made of sera from older infections in rabbits.

Attention is called to the fact that repeated stool examinations revealed that all of the infected animals began passing eggs between the thirtieth and forty-fifth days. The serum changes, therefore, seem to be concurrent with extensive oviposition. Our results are in agreement with those observed by Evans and Stirewalt (1957) in mice infected with *S. mansoni*, except for the fact that, whereas they observed the first changes in ionograms by the end of the twelfth week, in the present series marked changes were noticeable by the sixth week.

In human patients changes were noted similar to those observed by Fiorillo (1954) in *S. mansoni* patients. The changes, however, were less marked than those in the rabbits. This difference may be due, in part at least, to the fact that the rabbits were infected with a single heavy dose and studied for 6 months following exposure to infection, whereas human sera were obtained from individuals who lived in endemic areas and had probably become infected with *S. japonicum* repeatedly over a long period of time. It would be of interest to test sera from persons whose histories indicate a single exposure to cercariae, shortly after eggs have been passed in the stools, to determine whether dramatic changes occur, similar to those observed in rabbits. Attention is called to the fact that, whereas in normal rabbits A/G ratios of between 1.2 and 1.5 were obtained, the A/G ratio of noninfected persons in our series was 0.88. No explanation is available at the present time to account for the observed low albumen values.

The results of present studies in rabbits and human beings do not permit us to determine with any degree of certainty whether the marked increase in gamma globulin following schistosome infection is due primarily to liver damage or to the production of antibodies or to a combination of factors. Preliminary serological studies in rabbits indicate that the development of detectable antibodies to adult schistosome antigen takes place 30 to 45 days following exposure to infection and is, therefore, coincidental with extensive oviposition and the rise of gamma globulin. Studies are in progress in which the levels of serum transaminase are determined at various intervals following infection with *S. japonicum*. Study of the relations between serum transaminase levels, development of serological and cellular response, early oviposition and serum electrophoresis may throw some light on the relative importance of schistosome eggs, cercariae, and adults in the pathogenesis of disease due to *S. japonicum* and in the stimulation of host response.

SUMMARY

Successive samples of serum of rabbits infected with *S. japonicum*, and of uninfected controls, were fractionated by paper electrophoresis and changes were observed for a period of seven months. In the early stages of the disease, before

deposition of eggs, no notable changes in the serum-protein fractions were observed. However, beginning with early oviposition a marked increase in total proteins was observed. At this time there were also a sudden relative decrease in albumin and increase in gamma-globulin fractions which remained essentially unchanged up to the end of the experiment, 6 months following infection. Fractionations of sera from persons with proven schistosome infection showed significant increases in total protein and in the relative proportions of alpha-2, beta and gamma-globulin fractions, with a corresponding decrease in the relative proportions of albumin.

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STUDY OF A NATURALLY OCCURRING POPULATION INTERMEDIATE BETWEEN *CULEX P. PIPIENS* AND *C. P. QUINQUEFASCIATUS*[1]

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INTRODUCTION

Previous studies have indicated that *Culex p. pipiens* L., occurs in temperate regions but is replaced by *C. p. quinquefasciatus* Say in warmer regions. It has also been found that forms morphologically intermediate between the two subspecies occur in areas where the two come together (Bekku, 1956; Barr, 1957). The present study was designed to follow morphological changes in such an intermediate population over a period of time in order better to understand the dynamics of intergrading forms.

MATERIALS AND METHODS

Most of the work was done on a naturally occurring anautogenous population in Lawrence, Kansas. The population is made up predominantly of *C. p. pipiens*, but with a strong admixture of intermediates and some *C. p. quinquefasciatus*. For purposes of comparison a colony of *C. p. quinquefasciatus* was started with larvae from Montgomery, Ala., furnished by Dr. R. W. Chamberlain in April, 1956. Dr. A. R. Barr furnished specimens of adults and larvae of the typical form from Minnesota and New Jersey. Dr. J. N. Belkin of the University of California at Los Angeles supplied eggs of an autogenous strain ("*molestus*") from Los Angeles County (Malibu Beach) from which a colony was established. This colony, as will be shown later, is peculiar in that it is composed primarily of intermediates but is nevertheless autogenous. This is believed to be the first report of an autogenous strain of *C. p. pipiens* which is not morphologically similar to the typical form.

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The most reliable means of differentiation between *C. p. pipiens* and *C. p. quinquefasciatus* is a measurement of the male terminalia, *i.e.*, the DV/D ratio (Sundararaman, 1949), which compares the extension of the ventral arms of the phallosome laterally of the dorsal arms, to the distance between the dorsal arms. For a detailed explanation of the measurement as used in this work see Barr (1957). Typical *C. p. pipiens* males have ratios less than 0.2 and *C. p. quinquefasciatus* more than 0.4 (usually more than 0.5). Specimens having ratios between these values are intermediate.

Larvae were mounted in balsam, as were male terminalia. Most of the slides were prepared with two larvae on a slide; however, some slides had only one larva. The method of mounting may affect the siphonal index in that flattening of the siphon may occur, especially when larvae are mounted individually. When two larvae are mounted on a slide there is less likelihood of flattening. The siphonal index is the ratio of the length to the width of the siphon. The length of the siphon was measured along the uninterrupted dorsal surface and the width was measured at the widest place, which was usually at the base. The measurements for the siphonal index were done with a dissecting microscope having a grid in one ocular. All other measurements and counts were made with a binocular compound microscope.

The observations made on larvae, other than the siphonal index, were counts of the numbers of branches in the siphonal tufts, the numbers of branches in the pentad hairs 1, 3, and 5, and the numbers of pecten spines on the siphon. The siphonal tufts were numbered with the outermost as number one. Only the usual number of tufts (four pairs) was counted in this study. Larvae with only three pairs of tufts were found rarely but a number of individuals had a fifth tuft on one or both sides of the siphon; an occasional specimen had a sixth tuft on one side. The pentad hairs were numbered from one to five beginning dorsally.

REARING PROCEDURES

The colonies were kept in wooden-framed, cloth-covered cages with a glass top. These cages were cubical, measuring 16 inches on a side. Adult mosquitoes were regularly supplied with raisins and usually with 10 per cent honey solution (or sucrose). It appeared that more eggs were obtained when both raisins and honey were supplied than when either alone was used.

The first colony of "intermediates" (from Lawrence, Kansas) was kept in a cage of similar construction to the cages described above, but measuring 2 x 2 x 3 feet high, with a glass top and a glass observation window across the top third of the front. A guinea pig in a standard, laboratory-type, small-animal cage, provided a continuous source of blood, except for short periods when the animal cage was removed for cleaning. After about 6 months this colony was transferred to a small cage and fed on human blood. The other colonies were in the smaller cages described above and were fed on the author's arms. Adults of the "*molestus*" strain were fed only raisins and honey.

The cages were kept in an insectary at a temperature of 72 ± 3°F, a relative humidity of about 85 ± 5%, and were illuminated for 16 hours daily (4 a.m. to 8 p.m.). The number of individuals in the colonies ranged from about 100 to 3,000 or more. The females usually outnumbered the males considerably after the first week or so of colonization. The life-span of the male is much shorter than that of the female. There was no great fluctuation of the population size most of the time. Larvae were fed rabbit pellets (containing mostly alfalfa) and were kept at a temperature of 76 ± 5°F; the pans were continuously aerated. Larvae from individual egg rafts were reared with aeration in small finger bowls.

RESULTS

DV/D ratio. The colony started with mosquitoes from Lawrence, Kansas in the fall of 1955 has shown an interesting increase in the DV/D ratio over at least half of its period of existence (Fig. 1). The first sample, (n = 58) which was taken in October from field-collected mosquitoes with which the colony was started, had a mean DV/D ratio of 0.12; range −0.06 to 0.60 and SD[3] 0.14. The second sample (n = 17) from the colony, in December, 1955 had a mean DV/D ratio of 0.15; range 0.02 to 0.67, and SD 0.17. In succeeding samples the mean DV/D ratio has remained 0.24 (fourth month) or above. The graph of the mean DV/D ratio (Fig. 1) shows a rapid increase to 0.35 about 3 months after the first sample. Later samples have shown fluctuations about this value, with considerable variation. The lowest mean ratio after the fourth month was 0.25 and the highest 0.46. The samples from this colony ranged in size from 7 to 58 individuals; most contained more than 20 individuals.

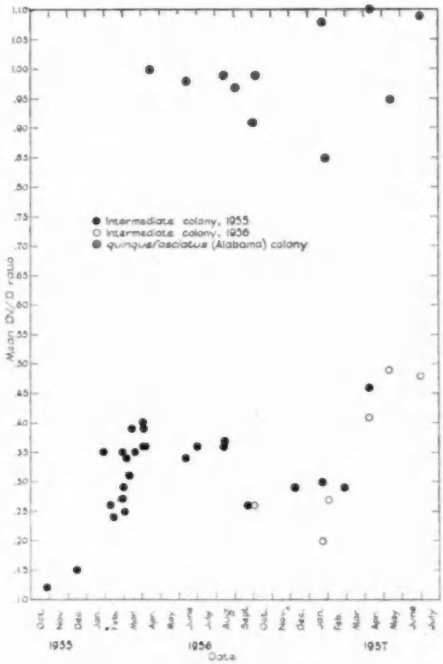

FIG. 1. Mean DV/D ratios of samples from *Culex p. pipiens* colonies

To determine if this increase in the DV/D ratio in colonies could be repeated, a second colony (Fig. 1) was started with mosquitoes collected from the same breeding place in October of 1956. The mean DV/D ratio of the sample taken at this time was 0.26. A second sample of the colony, 3 months later, had a ratio of 0.20. The ratio increased to 0.49 in May, 1957 and 0.48 in July, 1957. This is somewhat higher than the means of any of the samples from the first

[3] Standard deviation of the frequency distribution.

colony; the mean ratios of the stocks from which the two colonies were founded, however, were somewhat different (0.12 and 0.26 in 1955 and 1956, respectively). The samples from this colony contained 18 to 65 individuals.

The means of the DV/D ratio of the *C. p. quinquefasciatus* (Fig. 1) colony from Alabama have remained high. The lowest mean was 0.85 in January, 1957 and the highest 1.10 in April, 1957. There have been 11 samples from this colony, consisting of 6 to 94 individuals. The mean DV/D ratio of this colony has remained in the range of *C. p. quinquefasciatus*, the lowest ratio of an individual being 0.60 and the highest 2.00. There is considerable variation in the means of samples of this colony but they appear to fluctuate around an average value.

There have been two colonies of "*molestus*". The first was started in November, 1956 and flourished for about a month and then quite suddenly died out. The mean DV/D ratio of the first sample of males was 0.29. Three subsequent samples had mean ratios of 0.35, 0.28, and 0.25. The lowest ratio of an individual was 0.12 and the highest 0.55.

The mean DV/D ratio of the first sample of males of the second colony was 0.31. Two subsequent samples were taken at about 2-month intervals and the means were 0.29 and 0.31. The overall range for this colony was 0.15 to 0.67. The samples from these two colonies ranged in size from 13 to 74 individuals.

SEASONAL CHANGE IN THE DV/D RATIO

Samples of males were reared from immature mosquitoes collected in a single breeding place in Lawrence throughout the breeding season of 1956 (Fig. 2). The samples in this group contained from 12 to 51 individuals (all but three contained more than 20 individuals). The first sample was collected July 15; it had a mean DV/D ratio of 0.07, range 0.00 to 0.21. Another sample from this same batch had a mean of 0.09, range −0.03 to 0.22. Subsequent mean ratios of samples from this breeding place are all in what is considered the *pipiens* range (less than 0.20) until the collection of October 2, which had a mean of 0.26. This value is in the range of intermediates between *C. p. pipiens* and *C. p. quinquefasciatus*. With the exception of two samples (mean 0.08 on October 13, mean 0.16 on November 14) all the means of samples taken after October 2

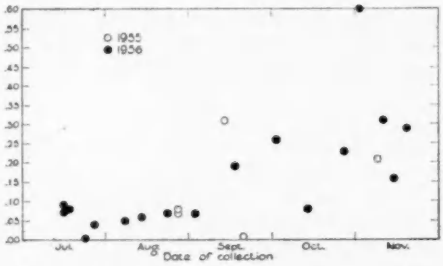

FIG. 2. Mean DV/D ratios of field-collected samples of *Culex p. pipiens* from Lawrence, Kansas.

are in the intermediate range, and one (0.60 on November 2) in the range of *C. p. quinquefasciatus*.

Similar data were collected in 1955. (Fig 2) but gave inconclusive results because of the small number of samples.

INDIVIDUAL EGG RAFTS

The population mentioned above was also sampled by collecting egg rafts throughout the breeding season of 1956 and rearing the progeny of each raft separately. The mean DV/D ratios of samples of the individual rafts are based on 3 to 35 males; most samples contain 15 to 25. The mean ratios of all of these rafts are shown in Fig. 3. As can be seen from this graph the range in means becomes larger as the season progresses. This broadening of the range is due to the higher values, present later in the season, but not found earlier in the season. Data from a few rafts taken in 1955 are also shown in Fig. 3 although they are too few in number to be conclusive.

From a consideration of the variability in the progeny of a single female (*i.e.*, within a single raft) it is seen that the offspring of a female are rather similar to each other; there is usually not a broad range of ratios within the progeny of a female. Usually the progeny from a female are all *C. p. pipiens*, are *C. p. pipiens* and intermediates, or are intermediates and *C. p. quinquefasciatus*. It is unusual to find only *C. p. quinquefasciatus* or to find all three types in a single egg raft.

During the same period that the above egg rafts were collected, 32 were also taken in artificial containers close to the insectary. Of these, 29 produced males which had ratios in the *pipiens* range and three produced *C. p. pipiens* and intermediates. It appeared that the trend in these

collections may have been somewhat different from that in collections from the locality discussed above, which was about 2 miles away.

LARVAL CHARACTERS

Many differences between *C. p. pipiens* and *C. p. quinquefasciatus* larvae have been described although, for the most part, these differences have not been evaluated. In this study ten fourth-instar larvae of Minnesota *C. p. pipiens* were compared with ten specimens of Alabama *C. p. quinquefasciatus* and differences between the two were noted. These characters were then checked on ten larvae from the 1955 colony established with material from Lawrence, Kansas. Some characters were included which appeared not to differ in the two subspecies. The results of this comparison are shown in Table 1. The characters used are the number of branches in the pentad hairs and in the siphonal tufts, the number of pecten spines, and the siphonal index.

As can be seen in Table 1 the "intermediate" strain appears to be closer to *C. p. pipiens* in some respects (number of branches in the fifth pentad, and the first and second siphonal tufts), closer to *C. p. quinquefasciatus* in one respect (number of branches in the third pentad) intermediate in some respects (number of pecten spines, siphonal index and number of branches in siphonal tufts 3 and 4), and is different from both in the branching of the first pentad hair. All of these characters were examined on all larvae in the study.

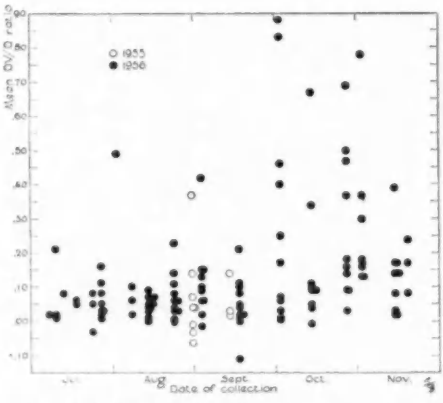

FIG. 3. Mean DV/D ratios of samples of males reared from individual egg rafts collected in Lawrence, Kansas.

SEASONAL DISTRIBUTION OF LARVAL CHARACTERS

The study of larval characters was done with larvae taken from the egg rafts from which males were reared for the study of the DV/D ratio. In most cases ten larvae from each raft were used. In these larvae the number of branches in the dorsal pentad hair (No. 1) appeared not to differ in the two subspecies. The mean number of branches in this hair for larvae from each raft is plotted against date of collection in Fig. 4. It can be seen that there is no consistent trend in the data although the values may be somewhat higher in the middle of the season than they are at the beginning or end of the season. Similarly, no trend can be seen in similar data for the middle pentad hair (No. 3); the higher values in midsummer are less clearly evident for this hair. Similar data for the ventral pentad hair (No. 5) show no trend and no higher values in the middle of the season. The data on pecten spines also show no conspicuous pattern although lower numbers of pecten spines were found only in early October. The data on siphonal index also show no trend throughout the season.

These characters apparently do not differ in the two subspecies and appear to remain fairly stable throughout the breeding season although there is a great deal of variability and random fluctuation. The siphonal tufts, on the other hand, are in contrast to these findings. The data for siphonal tuft No. 4 are shown in Fig. 5. It will be noticed that the mean number of branches

TABLE 1

Comparison of characters of fourth instar larvae of three strains of Culex pipiens

Strain	Branches in pentads			Pecten spines	Branches in siphonal tufts				Siphonal index
	1	3	5		1	2	3	4	
C. p. pipiens	4.8	7.9	5.2	14.1	2.9	2.7	3.0	3.1	4.6
Intermediates	4.1	7.2	5.4	12.7	2.7	2.4	3.5	4.0	3.7
C. p. quinquefasciatus	5.0	7.2	4.4	8.5	4.8	4.8	6.7	6.8	3.2

in this tuft is small early in the breeding season but becomes larger as the season advances. The range in mean values also increases as the season progresses, due largely to the appearance of high values later in the season. The pattern of values in Fig. 5 should be compared with the pattern in Fig. 3, which is based upon the genitalia of males from the same egg rafts.

The data for siphonal tuft No. 3 are very similar to those for siphonal tuft No. 4; they show exactly the same trend. The data for siphonal tufts No. 1 and No. 2 show this trend less clearly because of a smaller total range in variation.

Since egg rafts which produced larvae with siphonal tuft No. 4 highly branched also appeared to produce males with a high DV/D ratio, these two measurements for each egg raft were plotted in Fig. 6. This figure shows the relation between

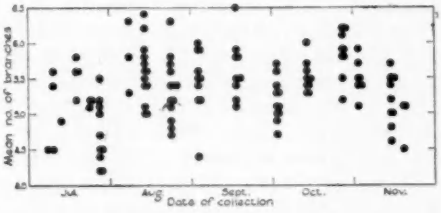

FIG. 4. Seasonal variation in the amount of branching of the dorsal pentad hair of samples of larvae from field-collected egg rafts.

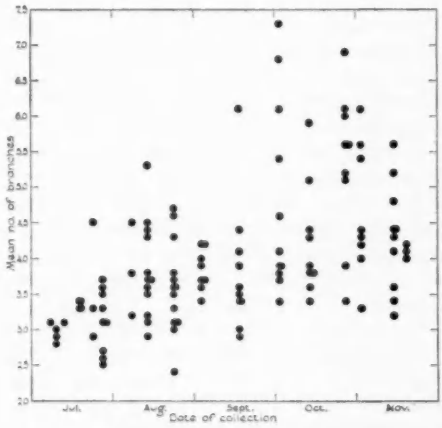

FIG. 5. Seasonal variation in the amount of branching of the siphonal tuft 4 of samples of larvae from field-collected egg rafts.

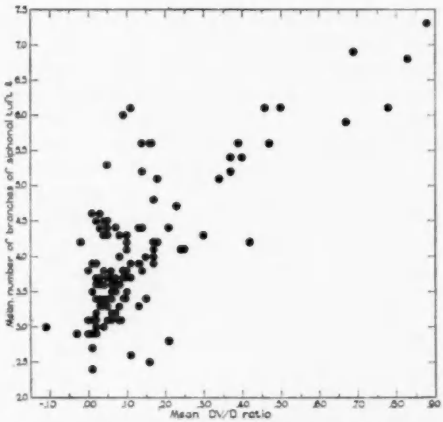

FIG. 6. Correlation of the DV/D ratio with the branching of siphonal tuft 4 in the progeny of individual females.

the branching of the siphonal tuft and the DV/D ratio for each raft. The relation is not a linear one since the ratio increases at a greater rate than does the branching of the tuft.

Not enough samples of larvae were taken from the laboratory colonies to detect changes in the amount of branching of the siphonal tufts.

DISCUSSION

It has been shown by Barr (1957) that *Culex p. pipiens* populations, in areas where both subspecies occur, may consist mostly of the two "parental" forms, with few intermediates (as in the St. Louis area) or may consist largely of intermediates (as in Sacramento County, California). This suggests that isolating mechanisms between the two forms exist but may become largely inoperative at certain times or in certain places. This study was designed to gather information on such isolating mechanisms.

Seasonal variation. In Lawrence, Kansas the *Culex p. pipiens* population appears to be fairly similar to the typical subspecies early in the breeding season as judged by the DV/D ratio (Figs. 2 and 3). In this locality the immature stages do not become common until July so it is difficult to secure data on early spring populations. In early July collections the DV/D ratios are in the range of typical *C. p. pipiens* and intermediates are uncommon. As the season progresses intermediates become increasingly common and eventually individuals similar to *C. p. quinquefasci-*

atus begin to appear. The data on mass rearings from the field populations (Fig. 2) do not show the source of variability in these later collections; they merely show an increase in the mean ratio in later collections. It can be seen in Fig. 3, however, that the reason for the rise in average ratio is due to the addition of larger values rather than the absence of smaller ones. Data for larvae from the same egg rafts show a similar trend (Fig. 5).

These data indicate that the Lawrence population tends to be similar to typical *C. p. pipiens* early in the year but becomes more variable and, on the average, more *C. p. quinquefasciatus*-like later in the season. This could result from an influx of *C. p. quinquefasciatus* (or intermediates), presumably from the south, or from a selection of the local population for traits possessed by *C. p. quinquefasciatus*. Although we can not confirm either of these hypotheses at the present time, the latter alternative appears unlikely because the range of variation is small early in the season and because typical *C. p. pipiens* occurs in large numbers throughout the entire breeding season.

It would appear that only individuals similar to *C. p. pipiens* are able to overwinter successfully in this area since the earliest collections in the season contain individuals of this type. The apparent inability of *C. p. quinquefasciatus* and intermediates to overwinter successfully could be physiological or behavioral. The data do not indicate when and in what manner selection against *C. p. quinquefasciatus* takes place. Figures 2, 3, and 5, as well as others not shown, indicate an absence of *C. p. quinquefasciatus*-like characters at the end of the breeding season which suggests that selection against *C. p. quinquefasciatus* may take place in the fall, before winter sets in. The data, however, are not conclusive on this point.

It appears from the above that *C. p. quinquefasciatus* is prevented from colonizing northern regions because of its inability to overwinter successfully in those regions. It is not yet understood why *C. p. pipiens* is not able to invade southern regions successfully. Farid (1949) has suggested that *C. p. pipiens* may be unable to mate successfully at elevated temperatures.

Colonization. When colonies of the local form were established it was found that the DV/D ratio increased within a few generations (Fig. 1). This indicates that the colony became less like *C. p. pipiens* or more like *C. p. quinquefasciatus*. The colonies were selected for feeding on human blood and were unavoidably selected for mating in a small space. Since *C. p. quinquefasciatus* appears to feed more readily on mammals than does *C. p. pipiens* and also usually requires less space for mating, it was not surprising to find that the colony became more like *C. p. quinquefasciatus* although this result had not been expected. It is unfortunate that more larval samples were not taken from the colonies to determine whether the larvae also showed a trend toward becoming more like *C. p. quinquefasciatus*.

Larval characters. Although several larval characters have been used to separate *C. p. pipiens* and *C. p. quinquefasciatus* it appears that few are useful for this purpose. The siphonal index is one of these which appeared valueless in the present study. The branching of the basal siphonal tufts proved to be the most generally useful in this study although the differences between the two forms are quantitative; individual specimens cannot be determined with certainty.

The branching of the basal siphonal tufts is fairly well correlated with the DV/D ratio of the male terminalia as can be seen in Figs. 3, 5, and 6. It appears that the seasonal variation in branching of the siphonal tufts is not an environmental effect since the same trend cannot be seen in the pentad hairs and since this change is correlated with changes in the male terminalia. This character should be further evaluated in other areas.

SUMMARY AND CONCLUSIONS

The major part of this study is based on a naturally-occurring, anautogenous population of *Culex p. pipiens* in Lawrence, Kansas. This population is composed predominantly of the nominate subspecies, but many intermediates and a few *C. p. quinquefasciatus* (morphologically speaking) are present. Comparisons were made with anautogenous colonies from Alabama (*C. p. quinquefasciatus*) and Lawrence, Kansas (intermediates), an autogenous colony ("*molestus*") from California, and with typical *C. p. pipiens* material from Minnesota.

The autogenous colony is composed mostly of intermediates. It is believed that this is the first report of a naturally-occurring, autogenous strain of *Culex p. pipiens* which is not morphologically similar to the typical form.

The characters used for study were the DV/D ratio of the males and the number of branches

in the pentad hairs (1, 3, and 5) and siphonal tufts, the siphonal index, and the number of pecten spines on the siphon of the larvae.

The two colonies established with material from the Lawrence population have shown an increase in the DV/D ratio since colonization. This shift from a form more like *C. p. pipiens* to one intermediate between *C. p. pipiens* and *C. p. quinquefasciatus* is probably produced in the laboratory by selection favoring *C. p. quinquefasciatus*.

One breeding place was studied in detail to determine the seasonal morphological variation of the local population. The mean DV/D ratio in the male terminalia of samples from this breeding place increased throughout the breeding season. From individual egg rafts it could be seen that this increase in the mean ratio was largely due to the adding in of higher values later in the season which were not present earlier in the season. This probably indicates an influx of individuals which are more like *C. p. quinquefasciatus* from other areas, presumably from the south.

Analyses of the variation in the branching of the siphonal tufts of larvae showed the same trend seen in the male terminalia. There appears to be a rather high correlation between the DV/D ratio of the male terminalia and the amount of branching in the basal siphonal tufts of larvae of this species in the Lawrence area.

The results of this study are in agreement with the view that *Culex p. pipiens* and *C. p. quinquefasciatus* are biological entities which intergrade in the field and thus satisfy the classic requirements of subspecific status.

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THE LARVAL HABITATS OF *AEDES POLYNESIENSIS* MARKS IN TAHITI AND METHODS OF CONTROL

DAVID D. BONNET[1] AND HERALD CHAPMAN[2]

INTRODUCTION

Effective measures for the control or eradication of a mosquito species require that information be available on the locations in which the larvae of the species will be found. Many persons unfamiliar with the problems of mosquito control are surprised when they learn of the great variety of habitats which have been utilized by mosquitoes as breeding places.

Although several workers have tried various classification schemes, no single system has proved adequate as a means of clearly separating the types of water or locations where the larvae of the different species will occur. In a general way, however, certain species are restricted to swamps, grassy ponds and ground pools while others are found only in isolated locations such as tree holes, rock holes, or crab holes, etc.

Bates (1949) has discussed the problem of the classification of larval habitats in detail.

Extensive studies on the bionomics of *Aedes polynesiensis* Marks are being made as part of the investigations on methods of filariasis control in Tahiti. This work has been part of the cooperative program between the Pacific Tropic Diseases Research Project, Department of Infectious Diseases, School of Medicine, University of California, Los Angeles, and L'Institut de Recherches Médicales, Papeete, Tahiti.[3]

The role played by *Aedes polynesiensis* in the transmission of nonperiodic *Wuchereria bancrofti* has been reported by numerous workers in the South Pacific, including Bahr 1912, Buxton 1927, Byrd *et al.*, 1945, Galliard *et al.*, 1949, Beye *et al.*, 1952, Jachowski and Otto 1954, and Rosen 1955.

Bionomic studies with observations on the places where the larvae of this species may be found have been given by Buxton and Hopkins 1927, Byrd *et al.*, 1945, Beye *et al.*, 1952, Jachowski 1954, Rosen and Rozeboom 1954, Rosen 1954, Ingram 1954, Laird 1955, and Peterson 1956.

The taxonomic separation of *Aedes polynesiensis* from *Aedes pseudoscutellaris* by Miss Marks in 1954 has complicated a review of the literature for descriptions of larval habitats. However, as it is believed that *A. pseudoscutellaris* (*sensu stricto*) is found only in Fiji, the "*Aedes pseudoscutellaris*" mentioned by the above authors for Samoa, Cook Islands and Tahiti can be referred synonymously to *Aedes polynesiensis*. A further complication is the recent description of *Aedes upolensis* Marks 1957, from Samoa, which has been confused with *Aedes polynesiensis*.

LARVAL HABITAT OF *AEDES POLYNESIENSIS*

Although no simple classification system of larval habitat has proved completely satisfactory, it is useful to tabulate the types of locations where a particular species has been found since each type may require a distinct method of elimination or control. As *Aedes polynesiensis* is known to utilize a great variety of both natural and artificial water-holding containers, it is convenient to separate the larval habitat into two categories:

A. Natural Breeding Sites

Natural breeding sites include all containers found in nature that are not primarily the result of human activity and which hold rain water for a period of time sufficient to produce adult mosquitoes.

1. *Rock holes.* There are many holes in the lava formations in Tahiti. These hold rain water and serve as typical, permanent, natural, larval habitats. Rock holes may vary from a few ml to 1 L or more in capacity. Most rock holes in Tahiti are small, but may be quite numerous in the fractured, vesicular, amorphous basalt found in certain areas.

Most rock-hole areas are in the mountains away from human habitation and are continuous

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[3] Aided by U. S. Public Health Service, Grant G-3811.

sources of mosquitoes owing to the more frequent rainfall at the higher elevations. Larvae are not found as regularly in the rock holes along the coastal plains as these containers are subject to periodic drying. This does not destroy the resistant eggs which are usually present but does slow adult production. Fortunately, with the clearing of land and the removal of rocks, few houses are subject to mosquitoes from this source. For those rocks that cannot be moved, the application of concrete to the holes will permanently eliminate these as mosquito producers. At present, there is no known practical method of eliminating mosquito breeding in rock holes in the rugged mountainous areas, and they will continue to be a steady source of adults which will attack people in the mountains and higher valleys.

In spite of the frequent breeding of this mosquito in natural containers in the mountain areas, the short flight range of *Aedes polynesiensis* (Byrd *et al.*, 1945; Jachowski 1954) permits reduction of the mosquito population along the coast where most of the inhabitants reside. However, from this constant reservoir, mosquitoes will reinvade the coastal areas should control be relaxed after successful mosquito population reduction has been accomplished.

2. *Fallen leaves.* The bases of coconut fronds, the boat-shaped bracts of the coconut flower and the leaves of various common plants, including the breadfruit and banana, frequently hold rain water.

Mosquito-breeding in fallen leaves occurs most frequently in uncleared, overgrown, coconut land and in areas at the foot of the mountain where forest and plantation mingle. In such areas, where rainfall is frequent and evaporation reduced because of forest shade, leaves may hold water long enough for complete mosquito development. In general, control is obtained by clean-up, including destroying or turning over. The Tahitian people pick up and burn leaves falling on their own property. Unfortunately, in some locations closely adjacent properties of different ownership are badly overgrown and neglected, resulting in nearby foci of mosquito breeding.

3. *Rat-eaten coconuts.* Tree or roof rats (*Rattus rattus* Linn.) in coconut trees in Tahiti create an important natural source of water containers from coconuts. The rats chew a circular hole in the young, nearly mature coconut by which they enter the nut and consume the meat. Later, the nut falls to the ground and rain water fills the hollow shell. It has been estimated by Bambridge (unpublished data) that approximately 35 per cent of the copra crop in Tahiti is destroyed by rats in this fashion. Iyengar (1957) records that 50 to 80 per cent of the coconut crop in the Cook Islands is destroyed by rats.

Since the coconut is the most important economic product of Tahiti and the coconut tree is planted extensively along the coastal belt of the island, where the majority of the human population lives, the rat-eaten coconut is a problem of great magnitude. Both Edgar (Beye *et al.*, 1952) and Rosen (1954) have reported the rat-eaten coconut to be the most important single source of breeding for *Aedes polynesiensis* in Tahiti.

Further observations have verified the abundance of rat-eaten coconuts, but experimental evidence indicates that it must be considered a transient or impermanent source of mosquitoes.

Tests with rat-eaten coconuts have shown that the rain water collected is not acceptable to *Aedes polynesiensis* until the coconut kernel has become completely destroyed and the initial putrefactive processes completed. The time required varied from 3 weeks to 3 months depending upon temperature and moisture conidtions, as well as on the initial amount of potentially putrefactive material remaining in the coconut. The ageing process is accelerated in moist areas and the rat-eaten coconut becomes a source of mosquitoes sooner than in drier and hotter areas. Other rotting processes, such as those due to fungal, bacterial and ground fauna activity, are also accelerated in the wet areas resulting in the ultimate destruction of the husk and the shell to the point where the coconut will no longer hold water and it ceases to be a mosquito breeder.

Thus the rat-eaten coconut as a source of mosquitoes is in a dynamic equilibrium between (a) the rate of production by rats, (b) the rate at which rat-eaten coconuts become suitable for mosquito larvae, and (c) the rate at which they are eliminated by rotting or removal.

Control procedures are based upon (a) reduction of the rat population, (b) banding of coconut trees with rat guards to reduce attacks

on coconuts in the trees, and (c) clean-up and destruction of the rat-eaten coconuts.

4. *Tree holes.* The problem of tree-hole breeding in Tahiti has been discussed in an earlier paper (Bonnet and Chapman 1956).

Several species of trees have large buttressed trunks (i.e., Mape, *Inocarpus edulis*), aerial-root formations (i.e., Banyan, *Ficus* sp.), or limb-crotch formation (i.e., Mango, *Mangifera indica*) that hold water suitable for mosquito breeding. Other trees (i.e., Guava, *Psidium guajava*; Burau, *Hibiscus tiliaceus*) have a tendency to form rot holes where limbs are broken off, either naturally by wind action, or by man.

Although many tree holes are found in nature, often they are caused by human activity. This is particularly true in the case of the breadfruit tree, where the top is cut, causing a rot hole to develop; or where steps are cut in coconut trunks.

Bamboo is a frequent source of breeding for *Aedes polynesiensis*, particularly in the cut stumps. Occasionally high winds break and split the internode allowing rain water to accumulate. Rats, on rare occasion, gnaw a hole permitting rain water to enter and accumulate at the nodal septum.

Rot holes in trees close to human habitations are one of the major permanent sources of the *Aedes polynesiensis* adults in Tahiti. For the control of filariasis transmission, it is important that these be totally eliminated.

The control of mosquito breeding in tree holes is complicated by their inaccessibility, number and permanence. Various methods of control including cutting out, filling with cement or tar, chemical treatment, etc., have been partially successful. A method, recently described (Bonnet and Chapman 1956), of planting a fern or similar plant in the tree hole, has shown promise of eliminating mosquito breeding in preliminary trials. Bamboo stumps may be eliminated as mosquito breeders by sawing or slicing the stem 0.25 inch above the node on one side and slanting toward the node, care being exercised not to split or break the septum. This allows the water to drain completely and not only prevents mosquito breeding but also stops the rotting of the node.

5. *Crab holes.* Holes dug by the land crab, *Cardisoma carnifex* Herbst, are a prominent feature of the flat coastal areas of Tahiti and adjacent islands and are a common breeding and resting place of *Aedes polynesiensis*. This breeding site has been frequently overlooked owing to the difficulties of demonstrating the presence of larvae. It is now recognized that these crab holes are a source of primary importance.

Efforts to recover larvae by excavation, flooding or aspiration with a rubber tube have been only partially successful and the evidence for the importance of crab holes must be considered indirect.

The following evidence can be cited: (a) A small islet of about 4 acres, approximately 1.5 miles from the main island of Tahiti, regularly showed the presence of adult *Aedes polynesiensis*. A thorough examination of the islet on several different occasions revealed no larvae or water-holding containers. However, adult mosquitoes, male and female, were observed emerging from the numerous crab holes present. (b) A residential property in Punauia had been thoroughly cleared for a distance of over 200 m in all directions and several intensive inspections revealed no tree holes, rock holes, or other container with water or larvae, yet the number of biting *A. polynesiensis* was large on all occasions. Immediate relief was obtained subsequent to the treating of all crab holes on the property with a 5 per cent DDT emulsion. (c) On several occasions groups of crab holes from which adults were seen emerging were examined and scrapings of dirt from 6 inches inside the opening taken. When these scrapings were placed in water in protected containers, 10 per cent showed first instar larvae of *Aedes polynesiensis* within 24 hours. (d) Third and fourth instar larvae and pupae of *Aedes polynesiensis* were recovered from crab holes in an area flooded by heavy rains, raising the water table to a point where the larvae could be readily captured. Because of the unusual habitat, it was first believed they might be a different species, but close examination revealed them to be identical with *Aedes polynesiensis* from other sources. (e) Adult blood-replete female *A. polynesiensis* have been observed entering crab holes, presumably as a hiding and resting area. This may account for the presence of eggs in the lining of the tunnel. It is believed that subsequent activity of the crab dislodges the eggs which then drop to the bottom of the hole, where water is usually present. There the development takes place.

Control of breeding in crab holes has been

effected by the direct application of insecticide to each crab hole. This is a time-consuming activity and the result is temporary. It is difficult to locate every hole. The elimination of the crab is more permanent and need be repeated only where new holes are being formed.

Successful control has been obtained by placing a few ml of carbon bisulfide in each hole and closing the hole with dirt. A less expensive method, using a bread bait impregnated with a 0.03 per cent Parathion suspension and introduced into each crab hole, has proved successful in preliminary trials. Six gallons of water containing 150 gm of 5 per cent wettable Parathion mixed with 12 loaves of bread diced into 0.5-inch squares was sufficient to treat over 1,500 crab holes in 25 man-hours. This method must be used with caution as the crab frequently ejects the bait from the hole, where it may be eaten by pigs, poultry, dogs, or other animals. When the crabs are poisoned they frequently emerge from their holes and may cause secondary poisoning by being eaten by domestic animals. These difficulties may be largely avoided by removal of exposed baits or dead crabs during the early morning of the first 3 days after the poison is set out, or by confinement of the domesticated animals. Closing the holes with dirt or sand after inserting the poison is also effective in avoiding secondary poisoning.

B. Artificial Breeding Sites

Artificial breeding sites include all containers, both permanent and temporary, which are the product of human activity and which hold water for a period of time sufficient for the production of adult mosquitoes.

1. *Cisterns, rain barrels, tubs, drums, etc.* In many islands of the Society group, apart from Tahiti, prominent appurtenances to the dwellings are the cisterns and tanks used to store fresh water. In some instances these tanks are well constructed, covered or uncovered concrete cisterns or, more frequently, a group of 55-gallon metal drums with the tops cut out, collecting rain water from a galvanized roof.

Control of mosquito breeding in these tanks and drums is best accomplished by total screening, which will not only keep out mosquitoes but also prevents contamination of the water by leaves, dirt or animals. Unfortunately, screening is costly and is rarely done in a permanent or satisfactory manner. Mosquito larvae may also be eliminated by the addition of a tablespoon or two of kerosene to the surface every two weeks without spoiling the use of the water for drinking purposes.

In large tanks or cisterns the introduction of larvivorous fish, such as *Gambusia* may be indicated.

2. *Bottles and tin cans.* The empty bottle or tin can is a source of mosquito breeding when exposed to rain and hidden in dense brush. This occurs most frequently when careless disposal of the empty containers occurs. Control of this breeding requires storage in covered containers or in a dry place pending total destruction by burial or dumping into the ocean. Except in the more populated urban areas, bottles and tin cans constitute a minor source of mosquito breeding compared to natural breeding sites.

3. *Automobile tires, machinery parts, stored junk, etc.* Although discarded automobile tires, machinery parts, and other stored junk do not occur frequently in rural Tahiti, whenever they are found and are exposed to rain, they become a local source of mosquitoes of a major magnitude. The recommended control includes temporary treatment with insecticides and subsequent storage under cover. Ideally, these materials should be discarded or destroyed so they will be unable to hold water.

4. *Canoes.* The importance of canoes as sources of mosquito breeding has been recognized for a long time. Buxton and Hopkins (1927) believed that canoes were responsible for the widespread distribution of "*Aedes variegatus*" by the Polynesian immigrants. The eggs of *Aedes polynesiensis* are resistant to drying, and observations in Tahiti have demonstrated the presence of eggs on the inner surface of dry canoes. The presence of dilute sea water does not inhibit egg laying by *Aedes polynesiensis*, which has been shown to prefer slightly brackish water (Wallis 1954). Experiments in Tahiti have shown that the addition of up to 10 per cent sea water has a beneficial effect upon laboratory cultures. The mosquitoes from these cultures were more robust and hardier than mosquitoes obtained from larvae in tap water cultures, utilizing dried yeast for food in each instance.

Although canoes or pirogues which are in frequent use rarely produce mosquitoes, canoes which have been hauled up on the beach and neglected for a period of time collect large amounts of rain water and become a source of

intense mosquito production. The habit of covering the canoes with coconut fronds to protect them from the heat of the sun aids by providing shaded moist conditions. The draining of the water from the canoes through a plug hole has been recommended, but in practice, it has been found, the people rarely leave their canoes with the drain hole open. This may be because they expect to utilize the canoe soon, or they wish to keep the wooden canoe in a moist state to avoid cracking.

Control of mosquito breeding in canoes can be obtained by periodic examination of all beach areas and treatment with a heavy dose of a residual insecticide of all canoes which have been abandoned. Such canoes can be identified by the lack of the outrigger or broken sides and by the accumulation of leaves and debris. In many instances, the canoes can be inverted, thereby, eliminating the water. In any event, it is desirable to treat with insecticides to kill the larvae which may later hatch from the eggs that are invariably present.

5. *Animal drinking pans.* The maintenance of drinking water for domestic animals creates a source of mosquito breeding that is difficult to control, particularly large containers which cannot be emptied easily such as horse troughs or the large iron kettles often used in cattle pastures. Smaller containers holding water for dogs, cats, chickens or other domestic animals can be regularly washed out, thus eliminating mosquito breeding. It is necessary to treat the water in the large containers with a small amount of kerosene at regular intervals. This must be repeated at regular intervals. It is possible that the introduction of a predacious insect, such as *Anisops* sp. or the copepod *Mesocyclops obsoletus* (Bonnet and Mukaida 1957) might give effective biological control in this restricted environment.

6. *Ant guards.* Many persons in Tahiti place tin cans filled with water under the legs of tables and food cupboards (*"garde manger"*) in order to prevent ants from getting at the food. These containers frequently contain the larvae of *Aedes polynesiensis.* It is suggested that the water be covered with kerosene or light oil to prevent breeding. One can also apply sufficient table salt to make a saturated NaCl solution, to which water may be added on occasion and in which the mosquito will not breed.

7. *Miscellaneous.* Among the locations where *Aedes polynesiensis* have been found occasionally are vertical metal pipes used as fence posts, clam shells, roof gutters and half coconut shells.

The last-mentioned item results from the grating of coconut meat or preparation of copra and is reported to be an important source of mosquito breeding in Fiji (Symes, personal communication). In Tahiti, the dried husks and coconut shells are commonly stored under the houses in a dry condition so that they can be used as fuel for the cooking fires, and this practice largely prevents the shells or husks from becoming water filled.

The control of mosquito breeding in these miscellaneous items cannot be given in general terms, as each will require a different method. Wherever possible, it is desirable to effect permanent control by total and permanent elimination of the water or the container.

DISCUSSION AND SUMMARY

A program of control directed against *Aedes polynesiensis* is not easily achieved because the locations where the larvae are to be found are so diversified. Consequently, one must direct attention not to a single habitat but to all receptacles that hold rain water.

As a guide to the control, one is tempted to make an evaluation of the containers in which breeding is found and place them in ranks in order of their importance. Although such a list has value in demonstrating the variety of habitats in which the larvae of *Aedes polynesiensis* may be found, the relative importance of each will vary from area to area. The importance or rank will depend upon relative abundance, volume, permanence, proximity to habitations or places of work or play, rainfall and suitability for breeding.

A somewhat more useful listing is to enumerate all types of containers capable of holding water (potential breeding containers). This is a census of the relative abundance of all types, breeding or not. A second listing giving the number of these containers with larvae of *Aedes polynesiensis* (actual breeding containers) enables one to determine the percentage of each available type in which breeding is actually occurring. Thus one determines the type of container where breeding is found most commonly. Jachowski (1954) has given a tabulation for *Aedes polynesiensis* in Samoa and the usefulness of this

type of survey in *Aedes* control in Hawaii has been reported by Bonnet (1947). These lists will have little value unless the sample is quite large and obtained during 1 or 2 years. This requires careful, systematic, continuous inspection of all areas on a cycle related to the length of time from egg to adult mosquitoes and can rarely be carried out because of the high cost of intensive inspections.

Further, it is obvious that such lists must be interpreted with care since no allowance is made for differences in quantitative production of mosquitoes from each container. A medium sized tree hole where the environment for the mosquito larvae is excellent for an entire year, may produce many more mosquitoes than several hundred rat-eaten coconuts. The latter may produce during only a few months of the year and then only during the period the contained water is not polluted. Yet small rock holes, by their very numbers, individually producing very few mosquitoes, may produce more than the tree holes when the production is summated.

This continuous production of mosquitoes is particularly important in the problem of filariasis transmission since filariasis requires frequent reinfection in order to maintain the disease in the human host (cf. Jachowski, *et al.*, 1951, 1952).

A large error may also be introduced if one overlooks the presence of eggs resistant to desiccation. One may have the impression from a survey that only a small percentage of rock holes, tree holes or crab holes are producing adult mosquitoes when, after a rainfall, 100 per cent or nearly so will have larvae which have hatched from the ever-present eggs.

At the present time, control of *Aedes polynesiensis* is dependent upon inspection of inhabited areas with the discovery and elimination of all waterholding containers. As an aid to the finding of the containers the cutting of brush and weeds for a distance of not less than 100 m from houses is recommended. Temporary containers such as bottles, tin cans, coconut shells, rat-eaten coconuts, and miscellaneous junk must be stored in dry places until destroyed by burial or dumping in the sea. Canoes should have rain water drained out regularly. Certain permanent useful containers such as cisterns, troughs, animal drinking pans, and ant guards cannot be eliminated and methods must be devised to make them unsuitable for mosquito breeding without destroying or interfering with their useful function.

It is believed that the most important sources of *Aedes polynesiensis* in Tahiti are the permanent, natural, breeding containers including tree holes, rock holes and crab holes. These containers serve to maintain mosquito populations through dry periods and act as "mother foci" to inoculate the temporary containers which cease to produce mosquitoes during adverse weather conditions.

Although the elimination of all containers which can breed *Aedes polynesiensis* is the ideal and should be attempted, it is not always possible to carry this out for practical reasons. A concentrated attack upon the permanent, natural, breeding containers should produce a greater degree of reduction per expended man-hour and therefore be less costly.

This review of the breeding locations of *Aedes polynesiensis* has been presented to emphasize some of the problems and difficulties encountered in the control of this medically important mosquito. It can be controlled, and its population reduced, if the persons engaged in the program not only use their imagination in finding the location of the larvae but also devise and enforce permanent methods of elimination or control.

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RELATION OF MEAN NUMBER OF FLEAS TO PREVALENCE OF INFESTATION ON RATS

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During a biological evaluation of a cooperative State-Federal program to control the flea vector, *Xenopsylla cheopis*, of murine typhus fever induced by *Rickettsia typhi* in rats and man, data came to hand from thousands of rats (*Rattus norvegicus* and *R. rattus*) and from hundreds of situations in 10 states during the years 1946 and 1047 (Mohr and Good, 1951; and Mohr *et al.*, 1953).

Some of the data were used by the U. S. Public Health Service Communicable Disease Center in Atlanta, Ga., and cooperating states in planning the control program. The data were complete enough for a study concerning the relation of the prevalence, or percentage, of infestation to the mean number of fleas per infested rat in several climatic zones. This relation is reported here.

METHODS

Specimens and data were taken in the usual manner in surveys of ectoparasites; the rats were caught alive in traps and then placed individually in cloth bags to prevent loss of ectoparasites. Collections were made during the period 1946–7 when flea prevalence and incidence of typhus in persons were both great. The rats were taken in various buildings before control operations in them were initiated.

In the biological evaluation here reported, the data are grouped by zones as follows: Zone I: limited area around New Orleans and most of Texas south of the latitude of San Antonio and east of Laredo; Zone II: the south coastal area of South Carolina, the southern third of Georgia, the southern quarter of Alabama, the southern third of Mississippi, all of Louisiana, and that portion of Texas south and east of Fort Worth and Dallas; Zone III: The northern portions of South Carolina, Georgia, Alabama, and Mississippi, most of Arkansas and north Texas excepting the Panhandle; Zone IV: North Carolina, and Tennessee.

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In general the northern and southern limits of these zones are indicated by isotherms in "Climate and Man" (1941) for average January temperature as follows: 55 to 60°F for Zone I; 47 to 55° for Zone II; 40 to 47° for Zone III and 35 to 40° for Zone IV. Actually, most of the data in Zone IV came from near the 40° area. The limits deviate only in that data from each county were treated as a unit and any county that was intersected by an isotherm was allocated to that zone in which most of the county lay.

In Zones I and II, most favorable for fleas during the period of study, the rats were taken in about equal numbers from outbuildings and buildings artifically heated during winter. In Zones II and IV, progressively greater numbers were taken from buildings that were heated artificially, chiefly because control and biological evaluation tended to be concentrated where human typhus cases occurred. In Zone IV, most were taken in heated buildings.

In recording the data (Fig. 1), the per cent of rats infested and the mean number of fleas per infested rat were calculated for each month and year of observation and recorded as circles for 1947 and circles with cross bars for 1946. A 3-month moving average then was calculated for each month and recorded as a dot. Where dots happened to fall on one of the circles mentioned above, the dots are enclosed in a large circle. The column to the right of each graph indicates the mean of the 12 sets of values. The number of rats examined for ectoparasites for each year and month is recorded under the appropriate graph. The calculations were made for months in which seven or more rats were examined.

OBSERVATIONS

Comparison of zones: In Zone I an annual mean of about 49 per cent of the rats was infested and an annual mean of about 6.3 fleas was present per infested rat (Fig. 1). An index number of 8 (49 ÷ 6.3) expresses the relation of the annual

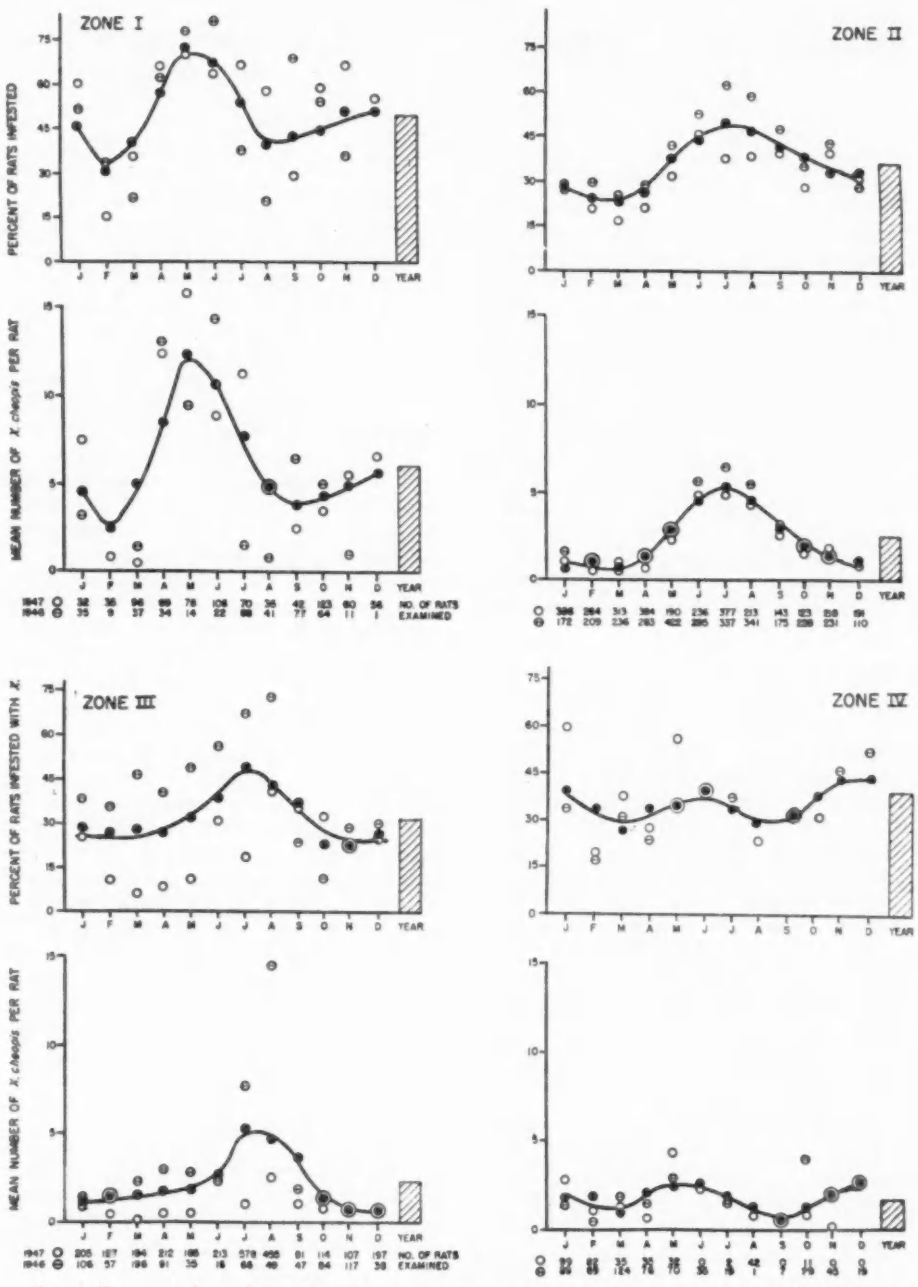

FIG. 1. Per cent of rats infested and mean number of *X. cheopis* per infested rat in four climatic zones of the United States, 1946–1947.

TABLE 1

Index numbers (per cent of rats infested, divided by number of X. cheopis *per rat) by month, in four climatic zones of the United States, 1946–1947*

Zone	Avg. Jan. temp., °F.	J	F	M	A	M	J	J	A	S	O	N	D
I	55–60	10	12	9	7	6	6	7	8	11	10	9	8
II	47–55	58	24	38	23	13	10	9	10	14	20	25	31
III	40–47	25	17	16	16	21	16	9	10	11	28	47	49
IV	35–40	21	18	27	17	14	15	17	25	24	26	23	19

mean number of fleas to the annual percentage of rats infested.

In Zone II an annual mean of about 36 per cent of the rats was infested and an annual mean of about 2.4 fleas occurred per infested rat. The annual index number for this zone was about 15.

In Zone III the annual mean per cent of rats infested was about 32 and the annual mean of about 2.4 fleas was present per infested rat. The index number for this zone was about 14.

In Zone IV the figures were 34, 1.8 and 19.

Differences between seasons: Indices for months, similar to the annual indices by zones, are presented in Table 1. For Zone I they indicate that the difference is greatest about February, with an index of 12, and great again in September or October, with indices of 11 and 10. These greatest differences occur after declines in flea populations end and an increase begins. The spread is least (6) in May and June when the decline begins. There are two periods of increase and decrease per year.

For Zone II the spread is greatest about January (index 58) and fairly great in December (index about 31). These relatively great differences occur during a period of general decline in flea populations. The spread is least (9) in July at the beginning of the annual decline. There appears to be only one period each of increase and decrease per year.

In Zone III the spread is greatest about December (index 49). This appears to occur at the beginning of an increase in flea population. It is least (9) in July at about the time of the peak population. There appears to be only one period each of increase and decrease per year.

In Zone IV the spread is greatest in March (27) and October (26) and least about May and June (14 and 15). Because only a relatively small number of rats were examined in this zone, particularly from June to December, the data are difficult to evaluate. There appear to be two periods each of increase and decrease per year as in Zone I.

There was a tendency for the annual smallest spread to occur later from Zone I to Zone III (May in Zone I and July in Zones II and III) and for the annual greatest spread to occur earlier in the winter (February in Zone I, January in Zone II, and December in Zone III. Apparently it occurred in October in Zone IV although there are indications that an equally great spread may have occurred in March.

DISCUSSION

Annual increase. The relation between the rate of increase of per cent of infestation and mean number of fleas per infested rat is of interest. In Zone I a strong upsurge in the number of fleas per infested rat began in February (Fig. 1) while the upsurge in prevalence of infestation was relatively less. Following the winter's adversities and possibly due to some hibernation on the part of the fleas, only about 30 per cent of the rats remained infested. About 2.5 fleas remained per infested rat. By May the mean number of fleas per infested rat had increased to about 12 or 13 and the prevalence of infestation only to about 73 per cent.

This difference in rate probably was due largely to the fact that a sharp increase first occurred in the flea population of those rats that remained infested and that the efforts of these fleas, aided by mating activity of the rats, resulted in increased distribution.

A similar differential upsurge also occurred in Zones II, III, and IV, but the relative rates were different, due possibly to differences in the nature of the climate and possibly to an increasing influence of artificially heated buildings on the flea populations.

Annual decrease. The annual decrease appeared to be initiated by a decline in the mean number of fleas per infested rat. Presumably those rats

in habitats least favorable to fleas lost their populations first and entirely. The mean number of fleas per infested rat dropped sharply during June in Zone I, during August in Zone II, and August in Zone III, followed by a moderately rapid decline in the prevalence of infestation.

In Zone IV the decline was not so sharp, either in the mean number of fleas per infested rat or in the prevalence of infestation. By September, both flea population and prevalence were on their second upswing for the year.

Relation to prevalence of murine typhus fever. Most cases of murine typhus fever in Zone I occurred in August about 2 months after the peak of prevalence of infestation. Most cases in Zones II and III also occurred in August but only about a month after the peak of prevalence. Most of the cases in Zone IV occurred in October and November during the second period of high prevalence, which apparently was greater than the first period and about as great as the single peaks in Zones II and III.

In Zone IV the prevalence of infection with the rickettsiae, and infestation by the vector flea seem not to increase sufficiently to cause considerable human infection until the heating of premises favors further increases in the number of fleas. The increase in prevalence may also be helped by the influx of susceptible rat populations coming to warmer winter quarters from their summer haunts in garbage dumps and sheds and outhouses. In these locations rats commonly have few Oriental rat fleas, and therefore are presumably relatively free of typhus.

SUMMARY

A comparison was made of the relation between mean number of *Xenopsylla cheopis* per infested rat, *Rattus rattus* and *R. norvegicus*, and the prevalence of infestation in four climatic zones in Southeastern United States. An increase of the mean number of fleas per infested rat, accompanied probably by increased activity of both rats and fleas in spring, induces an increased prevalence of infestation. A decrease of the mean number of fleas per infested rat, presumably most pronounced in habitats least tenable to fleas, induces a decline in prevalence. Two periods of increase and decrease occur in the northernmost and southernmost zones and only one each in the central zones. The second annual peak in the northernmost zone, most pronounced, seems to be induced by movement of rats into artificially warmed buildings in the fall. These are related to reported peaks and low points of prevalence of human typhus and presumably would be related to those of plague if it were present.

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A COMPARISON OF RESULTS OF HEMAGGLUTINATION AND DYE TESTS FOR TOXOPLASMOSIS IN A SURVEY OF TRINIDAD NATIVES

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Several serologic surveys have reported the prevalence of *Toxoplasma* dye-test antibodies or skin sensitivity among population groups in different geographic areas. A summary and interpretation of a number of these papers are given by Jacobs (1956a), and Feldman and Miller (1956) present a review of their own findings. A discussion of survey data in relation to climate is given by Gibson and Coleman (1958).

The original aims of the work to be reported here were two: First, previous studies on vegetarians had shown an apparently lower prevalence of positive reactions than might have been expected in a group of comparable age from the general population, but these vegetarian serums were of such diverse geographic origin that there was no really comparable control group (Jacobs, 1957). Since Trinidad has a sizeable population of East Indians, we hoped (in vain) that we might be able to obtain representative groups of vegetarian Hindus and other populations in the same locale for an adequate comparison. Second, we proposed to use these survey samples for a comparison of the dye test of Sabin and Feldman (1948) and the hemagglutination test (Jacobs and Lunde, 1957).

Through the courtesy of Dr. Wilbur G. Downs, of the Trinidad Regional Virus Laboratory, Rockefeller Foundation, we were able to obtain 121 serum specimens from Trinidad natives, including 26 from people of East Indian stock. We are grateful to Dr. Downs for his help, and for information on the food habits of the East Indian people. The serum specimens were sterile and were sent without preservatives.

On the basis of Dr. Down's information, we cannot be certain that all the East Indians adhered to the orthodox Hindu customs to the extent of shunning meat. Also, our sample of this group is, unfortunately, very small, and therefore our first aim has not been fulfilled. Most of this report is therefore devoted to the comparative data on the two serological tests.

METHODS

The dye test was performed using the technique described by Frenkel and Jacobs (1958). The main difference between this procedure and the procedure given by Feldman (1954) is the washing of the parasites in 10 per cent serum in Hank's solution in a siliconized tube and resuspending them in 80 per cent accessory-factor serum. This is done to remove soluble antigens which are present in the peritoneal fluid and which sometimes, at least, inhibit the dye-test reaction (Jacobs and Cook, 1954). The use of siliconized tubes for the washing procedure was first suggested by Eichenwald (1956), who also observed the same inhibition of the dye test by soluble antigens.

The hemagglutination test was performed as originally described (Jacobs and Lunde, 1957) except that our buffered saline solutions were prepared differently from those given by Stavitsky (1954). They were constituted as follows: pH-6.4-buffered saline (M/15 Na_2HPO_4, 26.7 ml; M/15 KH_2PO_4, 73.3 ml; NaCl 8.5 gm; distilled H_2O q.s. to 1 L); pH-7.2.-buffered saline (M/15 Na_2HPO_4, 72.0 ml; M/15 KH_2PO_4, 28.0 ml; NaCl 8.5 gm; distilled H_2O q.s. to 1 L).

The antigens used were pooled lysates of various harvests of *Toxoplasma*. Each lysate was first tested separately with a known positive serum to determine the maximum dilution at which it would give the same titer as obtained with the dye test. The lysates were then diluted to this maximum before pooling.

Serum specimens were tested at 4-fold dilutions: undiluted, 1:4, 1:16, etc. In the hemagglutination test the dilutions began at 1:4. The titer given is that of the original dilution of the serum, before addition of the other components of the reaction mixture.

RESULTS

Table 1 presents the over-all results of the survey, as revealed by both tests. There was a general increase in prevalence with increasing

age, as has been noted repeatedly since the first report of Feldman and Sabin (1949). It is noteworthy, however, that even in the first decade of life, 28 per cent of Trinidad natives acquire *Toxoplasma* antibodies. The agreement as to positivity or negativity between the dye and hemagglutination tests was 100 per cent, when only titers of 1:16 or higher were considered significant.

The distribution of titers obtained by both tests is given in Table 2. Here it can be seen that there was also good agreement between the tests as to the level of antibody demonstrated. There was 100 per cent agreement between the tests within a 16-fold (2-tube) difference in titer. Agreement within a 4-fold (1-tube) difference was over 90 per cent. In most cases, the discrepancies were due to slightly higher titers in the hemagglutination test.

The prevalence of antibodies among the 26 serums from East Indians was approximately the same as in the entire survey, with 50 per cent showing antibodies. An analysis by age group would be unprofitable, because the number of specimens is too small to yield statistically significant data.

TABLE 1

Hemagglutination and dye-test results on 121 serum specimens from Trinidad

Age group	No. tested	No. positive*		Per cent positive*	
		H.T.†	D.T.†	H.T.	D.T.
0–9	14	4	4	28.6	28.6
10–19	22	10	10	45.0	45.0
20–29	34	18	18	52.9	52.9
30–39	22	10	10	45.0	45.0
40–49	9	6	6	66.7	66.7
50 or >	20	18	18	90.0	90.0
Totals...	121	66	66	54.5	54.5

* Positive at 1:16 or >.

† H.T. = hemagglutination test; D.T. = dye test.

TABLE 2

Dye and hemagglutination test titers on Trinidad serum specimens

Dye test titers	No.	Neg.	Hemagglutination titers						
			4	16	64	256	1024	4096	16000
Neg.	50	49	1						
1:1	4	3	1						
1:4	1	1							
1:16	5			1	2	2			
1:64	17			2	6	7	2		
1:256	27				3	8	15	1	
1:1024	15				1	1	7	6	
1:4096	2								2
Total..	121								

DISCUSSION

Obviously, the prevalence of *Toxoplasma* antibodies on the island of Trinidad is very high. It approaches that found in Tahiti by Feldman, in the coastal areas of Guatemala by Gibson and Coleman (1958), and in other areas along the Caribbean coast by Biagi (1951).

The high prevalences of toxoplasmosis in these areas are given additional credence by the correlation between the dye and hemagglutination test results reported here. While various authors (Awad, 1954; Michalzik, 1953) have claimed that the dye test is not specific for *Toxoplasma*, other workers (Cathie, 1957; Jacobs, 1956) have not been able to show cross-reaction with other parasites. Ruling out cross-reactions, if the high prevalence of positive dye tests in tropical areas were due to an artefact, it would seem very unlikely that the hemagglutination test would respond in the same way.

At the present time, no explanation can be offered for the apparently high rate of transmission of toxoplasmosis in such tropical areas. Certainly it seems that any number of these locations would be suitable for studies on the means by which *Toxoplasma* can be acquired by both human beings and animals.

The evaluation of the hemagglutination test reported here shows that it may become a valuable tool in the diagnosis of toxoplasmosis. It certainly appears adequate for survey purposes. However, more work must be done on its usefulness in the diagnosis of the acute infection, where its ability to detect antibodies early in the course of the disease is the crucial point.

CONCLUSIONS

Excellent agreement as to positivity and negativity between the dye and hemagglutination tests for toxoplasmosis was obtained in a survey of 121 Trinidad natives. There was also close

agreement as to titer. The overall prevalence revealed was 54.5 per cent. Even in the first decade of life, a high prevalence of antibodies was found.

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HYBRIDIZATION OF *CULEX PIPIENS FATIGANS* WIEDEMANN FROM THE PHILIPPINE ISLANDS WITH AMERICAN STRAINS OF THE *CULEX PIPIENS* GROUP (DIPTERA: CULICIDAE)[1]

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The *Culex pipiens* group of mosquitoes has been receiving attention from several investigators because it serves as a convenient model for studies on the mechanisms of speciation in mosquitoes. One approach to elucidating the relationships between populations is to determine the degree of interfertility between them through cross-breeding experiments. The purpose of this paper is to present the results of such experiments with *Culex fatigans* from the Philippine Islands and several strains of North American *C. pipiens* populations.

MATERIALS AND METHODS

The Philippine strain of *C. fatigans* was first colonized by Dr. Louis C. LaMotte, from specimens collected on Luzon in the vicinity of Manila and Angeles. The colony, therefore, probably is a hybrid of the populations of the two localities. Strains of *C. pipiens pipiens* (anautogenous), and of *molestus* (autogenous) from Baltimore have been maintained in our laboratory for several years. A strain of *C. p. fatigans* from Galveston, Texas, was obtained from Dr. D. W. Micks.

Pupae were isolated in test tubes and the emerging adults were transferred to lantern chimneys according to sex. About 50 individuals of each sex were placed in the desired combinations in breeding cages 1 cubic foot in size. They were fed on chickens and sugar water. Eggs were deposited in a bowl of water kept inside the cage; the rafts were transferred individually to test tubes and observed for hatching. Records were kept of the numbers of rafts which were infertile, and the numbers which were fertile as determined by hatching of at least 20 per cent of the eggs. Infertile batches either failed to hatch entirely or, in a few cases, produced only one or several larvae. For each population, a number of rafts were treated as follows: within a few hours after hatching, the larvae were counted and transferred to white porcelain bowls. Each batch of larvae was placed in a single bowl and subsequently reared to the pupal stage in this bowl. Following hatching, the shells and unhatched eggs in each raft were counted with the aid of a stereoscopic microscope. The larvae were fed ordinary laboratory chow, and an effort was made not to overfeed. Scum was removed mechanically from the surface of the water whenever necessary. Records were kept of the number of pupae that appeared in each batch, and of the numbers of male and female adults which emerged. Pupae were transferred to test tubes, in which the adults emerged. Samples of these adults were used to establish F_2 and backcross colonies.

RESULTS

The numbers of egg rafts deposited, and the percentages of hatching, are presented in Table 1. In the first experiment, only 59 per cent of the rafts from the Philippine *fatigans* control hatched, which was much lower than the hatching in the experimental hybrid eggs. In the second experiment, the only obvious reduction in fertility was in the eggs produced by the Philippine *fatigans* female x *pipiens* male cross. Although there were only 12 rafts from this cross in Experiment 2, other observations, not recorded here, have shown the same results; only relatively few rafts were produced by this combination, and only a few hatched. However, the F_1 larvae which did hatch were entirely normal, and developed to completely fertile adults which produced vigorous F_2 progeny. The reduction in fertility of the F_1 eggs is evidently the result of a mating barrier between the Philippine *fatigans* females and the Baltimore *pipiens* males.

The purpose of determining the rates of hatching and of larval survival in individual batches was to determine whether partial sterility existed in eggs from a single female,

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and whether there was any reduced vitality in F_1 larvae. These data are presented in Table 2.

It should be noted that in some of the crosses fewer batches were reared through the larval and pupal stages than were recorded as fertile or nonfertile in Table 1. Thus, in the Gf x Gf cross of Experiment 1, 42 egg rafts were observed for fertility (Table 1), but of the 39 which hatched, 17 were observed through their development to the adult stage (Table 2). Since many batches from the various crosses were being reared simultaneously, it was not possible to include all those that hatched in these rearings. Those batches that were reared were selected at random. Hatching, pupation, and emergence are presented in each stage of each cross as a mean of the N number of survival rates; the standard deviation of these rates is included as a convenient means of showing the extent of variability between batches of a given cross.

In Experiment 1, 17 rafts from the Galveston control (Gf x Gf) totalled 3,823 eggs, giving a mean of 225 per batch. A total of 3,646 larvae hatched from these eggs. The per cent hatching among the 17 batches ranged from 83.0 to 100, with a mean of 95.9 per cent and a standard deviation of 6.1 per cent. Nine of the 17 batches were observed for subsequent development; a mean of 59.4 per cent of the larvae pupated; these 1,263 pupae produced 1,182 adults. The

TABLE 1

Fertility of egg rafts

Exp. No.	Cross* ♀♀ × ♂♂	Rafts	Hatched	Per cent fertile
1	Gf x Gf	42	39	92.9
	Mf x Mf	39	23	59.0
	Gf x Mf	24	21	87.5
	Mf x Gf	15	12	80.0
2	mo x mo	39	35	89.7
	P x P	8	7	87.5
	Mf x Mf	5	5	100.
	Mf x mo	10	7	70.0
	mo x Mf	53	49	92.5
	P x Mf	13	13	100.
	Mf x P	12	2	16.7

* Gf = *C. fatigans* from Galveston, Texas; Mf = *C. fatigans* from Luzon, Philippine Islands; mo = *C. molestus* from Baltimore, Maryland; P = *C. pipiens* from Baltimore, Maryland.

TABLE 2

*Summary of results of hatching and rearing of individual batches**

Exp. No.	Cross	Eggs				Per cent hatching of eggs				Per cent larvae pupating				Per cent adults from pupae			
		N.	t.n.	x̄	S.	N.	t.n.	x̄%	S%	N.	t.n.	x̄%	S%	N.	t.n.	x̄%	S%
1	Gf x Gf	17	3823	225	55	17	3646	95.9	6.1	9†	1263	59.4	29.0	9	1182	92.8	7.3
	Mf x Mf	20	3033	152	34	20	2764	91.0	19.0	20	950	37.3	30.1	15	846	89.7	10.7
	Gf x Mf	12	2371	198	65	12	2104	83.4	27.9	21	2632	63.0	24.3	21	2490	95.4	3.9
	Mf x Gf	12	2467	206	40	12	1759	73.5	21.2	12	1437	85.4	20.8	12	1403	97.8	1.9
2	mo x mo	12	760	63	17	12	678	89.3	15.7	12	509	77.3	14.0	12	493	96.9	4.1
	P x P	7	1295	185	42	7	1075	82.8	18.9	7	330	32.7	19.4	7	310	94.6	3.2
	Mf x Mf	5	1048	210	27	5	972	92.8	5.4	5	556	57.0	29.3	5	542	97.9	1.3
	Mf x mo	7	1447	207	35	7	1349	93.6	5.1	7	786	59.1	17.9	7	763	97.3	3.3
	mo x Mf	12	665	55.4	20	12	558	85.4	10.8	12	400	71.4	13.9	12	377	96.0	6.6
	P x Mf	13	2628	202	49	13	2456	90.9	14.5	13	1303	57.0	17.1	13	1286	98.9	1.4
	Mf x P	2	334	167	—	2	202	60.5	—	2	167	82.4	—	2	166	99.5	—

* N = Number of egg rafts or batches of larvae or pupae; t.n. = Total number of individuals in N number of batches; x̄ = Mean; S = Standard deviation.

† Differences in N. and t.n. between eggs, hatching, pupae, and adults are due to the fact that it was not possible to rear to adults all batches which had been counted as eggs and newly hatched larvae. In the Gf x Mf cross of Exp. 1, 21 batches of newly hatched larvae were counted for subsequent rearing, but in only 12 of these was it possible to count the eggs.

mean survival of the adults was 92.8 ± 7.3 per cent. It should be noted again that the per cent survival was not determined by 1,182 ÷ 1,263, but by averaging the nine survival percentages shown by the nine batches. In the Philippine *fatigans* control the mean of the larval survival rates was only 37.3 per cent; this low rate resulted from failure of five of the 20 batches to produce pupae. In the Gf x Mf F_1 cross, the mean of the hatching rates of 12 rafts was 83.4 per cent. Although both eggs and newly hatched larvae could be counted in only 12 batches, it was possible to count newly hatched larvae of a total of 21 batches, of which the mean survival to pupation was 63.0 per cent. This, as well as the survival of the Mf x Gf larvae, was greater than that of the parent strains.

In Experiment 2, the mean number of eggs for the *molestus* control was 63; these, as well as the eggs deposited by the *molestus* females of the mo x Mf cross, were autogenous, which explains the low numbers. In view of the non-reciprocal sterility often observed in crosses involving *molestus* (Rozeboom and Kitzmiller, 1958), it should be noted that the hatching and survival rates of the reciprocal Baltimore *molestus* x Philippine *fatigans* were about equal to one another and to those of the parent controls. The lowest larval rate was that of the *pipiens* control (32.7%). In general, survivals in the F_1 hybrids were equal or superior to those of the parent controls. The two rafts which hatched in the Mf x P cross produced 202 larvae, which developed normally.

TABLE 3

Sex ratios of adults

Exp. No.	Cross	No. ♀♀	No. ♂♂	Ratio ♀:♂
1	Gf x Gf	96	53	1.8:1
	Mf x Mf	404	442	0.9:1
	Gf x Mf	1113	1377	0.8:1
	Mf x Gf	673	730	0.9:1
2	mo x mo	254	239	1.1:1
	P x P	129	181	0.7:1
	Mf x Mf	237	305	0.8:1
	Mf x mo	359	404	0.9:1
	mo x Mf	183	194	0.9:1
	P x Mf	533	753	0.7:1
	Mf x P	93	73	1.2:1

In Table 3 a comparison is made of the sex ratios of the adults. It will be seen that with the exception of the rather small sample in the Galveston *fatigans* control, the sex ratios are essentially the same in all the populations, with the males usually slightly outnumbering the females.

From the results presented above, it seems clear that there is not a genetic barrier between *C. fatigans* of the Philippine Islands and North American *C. fatigans, pipiens,* and *molestus.*

DISCUSSION

In recent years there has come to be a rather wide acceptance of the *fatigans* and *pipiens* populations as representing subspecies of *C. pipiens sens. lat.* This is based upon the consistent success that has been obtained in crossing populations of the two subspecies from a number of localities throughout the world. The combinations which have been tried have been summarized by Rozeboom and Kitzmiller (1958). Observations by Sundararaman (1949), Barr (1957), and Bekku (1956) have shown that hybridizations in nature may be extensive. The populations involved in these studies have for the most part been from neighboring regions, such as northern and southern U. S. Roubaud (1956), on the basis of reduced hatching of F_1 eggs obtained from strains of *fatigans* from Brazzaville, Dakar, and Noumea, concluded that *C. fatigans* is actually a complex of species. This represents a view at the opposite extreme from that given above, and should give comfort to those who wish to retain *C. pipiens* and *C. quinquefasciatus* (*fatigans*) as true species on the basis of their slight morphological differences.

Speciation mechanisms involve geographic isolation, followed by gradual accumulation and accentuation of biological and genetic discontinuities, resulting eventually in reproductive isolation. Such reproductive isolation may be caused by differences in habits which prevent cross mating, or by true genetic intersterility. The present distribution of the *C. pipiens* (*sens. lat.*) populations is evidently the result of migration. Where the original divergence took place is unknown, but at present we seem to be observing a meeting of the two forms in many areas where environmental conditions are favorable to both.

The origin of the Philippine *fatigans* is unknown, but it would appear likely that this strain has been separated from the American populations for a long time. Yet this geographic isolation has not led to genetic barriers; as the cross breeding experiments herein reported have shown, the hybrid eggs were as fertile, with the one exception, as were those of the parent strains.

The lowered fertility in the Mf x P cross was undoubtedly due to a partial mating barrier which prevented successful insemination. The larvae which hatched from the few fertile egg batches in this cross, as well as those of all the other crosses, were as vigorous as those of the controls, and had survival rates which were comparable to those of the controls. All of the F_1 hybrids were tested further by allowing them to produce an F_2 generation, and by backcrossing to the parent strains. In all cases healthy colonies were established. To the writer, such results signify that the Philippine *fatigans* and North American *pipens* and *molestus* are potentially interbreeding forms, and that they represent subspecies, at most. The interfertility of *C. p. fatigans* from Nigeria and British Guiana, reported by Service (1956) supports the evidence from the Galveston x Philippine *fatigans* crosses that the widely separated populations of this subspecies are not taxonomically separable.

It must be recognized that within the broad specific limits of *C. pipiens* (*sens. lat.*), there is ample opportunity for the many populations to develop distinctive characters. Differences between *fatigans* populations may be observed in feeding and mating habits, responses to insecticides, and ability to transmit *Wuchereria bancrofti*. With more study many other biological and physiological peculiarities may be revealed. We have observed some of these to be under genetic control. Thus, although differentiation has not proceeded to the development of specific barriers, there appears to be an array of populations, more or less distinct in habits, biological characteristics, and presumably in genetic makeup, which fall within the two major subspecies of *Culex pipiens*.

In a recent paper, Mattingly (1957) has suggested that the North American *pipiens* is not the true *C. pipiens pipiens* of northern Europe. He questions the ability of the latter to hybridize with *fatigans*, and advances the hypothesis that the success of the hybridization experiments with the American *pipiens* is due to the intermediate character of this form. Barr (1957) has shown that *C. pipiens* in North America exists as a north-south cline, and I would hesitate to accept the northern populations of this cline as being an intermediate form. However, it is quite likely that further study will reveal physiological and to a certain degree, genetic differences between the northern European and northern American *pipiens*.

The systematic relationships between the *pipiens*, *fatigans*, *molestus*, and other populations belonging to this group of mosquitoes are still poorly understood, and no doubt there will be disagreement between those interested in the problem for some time to come. I have never observed, in my experimental crosses, high percentages of nonfertile eggs in hybrids that did not also occur from time to time in material from the parent colonies, nor have I recognized the occurrence of "lethal larvae" (Roubaud, 1954) in hybrid progeny. Thus, it would be difficult for me to accept Roubaud's (1956) statement that the possibilities of genetic mixing between *fatigans* and *pipiens* are extremely limited by reason of the great number of nonviable F_1 egg batches. On the other hand, we must take into account the observation by Barr that in some areas of overlap in the United States the two parental forms are more common than the intermediates. This indicates the existence of other isolating mechanisms, and indeed the present study, as well as that of Rozeboom and Gilford (1954), shows that there are differences in sexual activity which, under certain environmental conditions, would possibly function as a mating barrier.

The true relationship will be elucidated only after many more hybridization experiments and studies on comparative physiology, ecology, and genetics. To quote Mattingly (1957): " . . . we now know that these difficulties exist, and this is better than living in the aura of comfortable ignorance engendered by the use of categories of convenience." The very difficulties presented by this group furnish opportunities for contributions to understanding of mechanisms of speciation, which should apply not only to mosquitoes, but to the species problem in general.

SUMMARY AND CONCLUSIONS

Hybridization experiments with *Culex fatigans* from the Philippine Islands and North American strains of *C. fatigans*, *pipiens*, and *molestus*

showed that the Philippine mosquito is interfertile with the American populations. Low hatching in F_1 egg rafts from the Philippine female x *pipiens* male cross was probably due to reduced mating activity; larvae from the rafts which did hatch, and larvae from all other crosses, developed normally. Survival rates of the hybrid larvae and pupae were comparable to those of the parent strains. All F_1 hybrids produced vigorous F_2 and backcross colonies.

It is concluded that *C. fatigans* from Luzon in the Philippine Islands is conspecific with *C. pipiens*, *molestus*, and *fatigans* from North America.

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A COMPARISON OF METHODS FOR THE ISOLATION OF *TOXOPLASMA* FROM SUSPECTED HOSTS

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Although all parasitologists will acknowledge that the isolation of organisms in laboratory animals constitutes the best possible evidence of *Toxoplasma* infection, there have been few if any comparative studies to determine the best method of recovery of this parasite. Empirical procedures have been followed, due most likely to the lack of opportunity of testing the efficiency of different techniques in naturally infected animals.

The discovery that Memphis animals, particularly cats, had a high prevalence of infection, and that selection of animals on the basis of serological findings increased the proportion of infections found, made it feasible to attempt a comparative study without risking undue loss of time on uninfected animals.

Several methods of isolation have been practiced or suggested. These include the use of intraperitoneal and intracerebral injection of tissues from suspected hosts, followed by a varying number of blind passages. For instance, Feldman (1954) recommended the practice of both routes of injection, using three passages if parasites were not detected, and earlier, Jacobs *et al.*, (1952) used a rather complicated scheme of six "overlapping" passages, his use of this system being due entirely to the lack of knowledge at that time as to the best means for recovering parasites. Other workers (Erichsen and Harboe, 1953) suggested the use of cortisone to render animals receiving the inocula more susceptible.

More recently, workers in the field have used the dye test in conjunction with subinoculation procedures in an effort to render the isolation of parasites more certain and less laborious (Aagaard, 1956).

It was the purpose of our study to compare carefully, by simultaneous application, a number of the techniques which have been used, in order to work out an efficient and economical method for routine use in our laboratory.

METHODS

Mice were used as the laboratory animal of choice because of their high susceptibility and their freedom from spontaneous *Toxoplasma* infection. A few experiments were done with rabbits. Cats, obtained for the most part from the City Pound, constituted the naturally infected hosts. A previous study had shown that about a fifth of all cats from this source carry demonstrable infections (Jones *et al.*, 1957). During most of the study we used only cats with dye-test titers of 1:64 or higher, since previous work had indicated that demonstrable infection could be expected in half or more of cats thus selected.

Isolation was attempted only from brain tissue, as experimental work had shown that this tissue was most likely to contain chronic stages of the parasite, and a limited amount of work with naturally infected animals had indicated that the same is true for natural infections.

The cats to be used in the study were bled from the heart, usually under light, sodium-pentothal anaesthesia. During most of the study the cats with negative or low-titer positive dye tests were discarded. After dye-test results were available, the selected cats were sacrificed using either chloroform or a combination of sodium pentothal and chloroform.

The brain of the animal suspected of harboring *Toxoplasma* infection was removed intact to a sterile dish, and a 10-ml volume of tissue from several levels of the brain was added to 30 ml of saline (containing 10,000 units of penicillin and one mg of streptomycin per ml) in a graduated cylinder. The mixture was ground in a Waring blendor for 1 minute, then used for parenteral inoculations. Other bits of tissue taken from different levels were used in *per os* (p.o.) infection experiments.

For purposes of the study, several procedures for recovery of parasites were employed, as described below.

1. *Intraperitoneal (i.p.) inoculation with corti-*

sone followed by sacrifice of mice at 1- to 2-week intervals. Three or more mice were inoculated i.p. with 1 ml of brain suspension. Just after inoculation each mouse was given 5 mg of cortisone acetate intramuscularly in a hind leg. One mouse was sacrificed at the end of 1 week and another after 2 weeks. A preparation of peritoneal exudate, made by touching a cover glass to the exposed organs of the mouse, was examined in the fresh state, and Giemsa-stained smears of brain, liver, and peritoneal exudate were examined under oil immersion. Of course, any mice dying during the experiment were also examined as carefully as their condition allowed.

2. *Intraperitoneal inoculation as above but without cortisone.* An identical procedure was followed except that mice were given no cortisone.

3. *Intraperitoneal inoculation with cortisone followed by multiple passages in mice.* The original inoculation was made as in the first two procedures. Cortisone was given as in method 1. Passages from mouse to mouse were effected by grinding the brain of the donor mouse in a mortar with 2 ml of saline and injecting aliquots intraperitoneally into two uninfected mice, following which each mouse was given 5 mg of cortisone acetate intramuscularly. When mice were sacrificed for passage fresh and Giemsa-stained smears were examined for organisms.

Passages were made as follows, the scheme being modified from that of Jacobs *et al.*, (1952). One week after the original inoculation, the brain of one mouse was passed to two fresh mice; at the end of the second week, one of these mice was used for the inoculation of two third-passage mice. Four weeks after the original inoculation, the third-passage mice were reinoculated with the brain of the remaining first-passage mouse, and after a lapse of another week, the remaining second-passage mouse was passed to the third-passage group. The third-passage mice were sacrificed and examined 6 weeks after receiving the initial inoculation and 3 weeks after the final.

4. *Intracerebral (i.c.) inoculation.* Mice were inoculated i.c. with 0.02 ml of brain suspension using a 24-gauge, $\frac{5}{8}$-inch needle. Mice were sacrificed after 1 and 2 weeks as in procedure 1 described above. During a part of the study, some of the i.c.-inoculated mice were given cortisone, but the mortality from causes other than toxoplasmosis was so great in this group that it was discontinued.

5. *Intraperitoneal inoculation followed by dye test.* This method was adapted from that of Aagaard to which reference was made earlier. Mice inoculated intraperitoneally were sacrificed after 6 to 8 weeks and bled from the heart. Smears of brain were prepared for staining and the remainder ground in a mortar for inoculation. The blood sera were tested for their dye-test reaction. The brain smears were examined under the low-power objective of the microscope in either fresh or stained preparations. During part of the study, mice were bled from the orbital sinus using a capillary pipette with a rubber bulb. Dye-test results were thus available before sacrifice of the mice.

In some instances passages beyond the first were made but in others passage was discontinued when parasites had been detected, and no uniform procedure was used throughout the study.

6. *Feeding of tissue.* Tissue from different levels of the brain, approximately 2 ml in total volume, was placed with each of four mice in individual jars. Water was denied the mice and some mice were prestarved. Any tissue remaining after 24 hours was discarded, and records were kept of the amount consumed. Mice were bled after 8 weeks and dye tests were run. Brain smears were examined microscopically and in most instances brain tissue was passed to uninfected mice either orally or parenterally.

7. *Intraperitoneal inoculation of rabbits.* In a limited number of trials, two young rabbits which had been found dye-test negative, were inoculated i.p. with 5 and 10 ml, respectively, of brain suspension. Three weeks later they were again dye tested, and Giemsa-stained smears of brain, heart muscle, liver, spleen and peritoneal exudate were examined.

RESULTS

Comparison of single versus multiple passages (procedures 1 and 3). In all, 68 comparisons were made of the results of single versus multiple passage as outlined under procedure 3 of the methods section. Seventeen infections were detected, of which 16 were seen on the first passage. Thus, the use of three passages led to the detection of only a single additional infec-

tion. It is possible, of course, that a large number of passages might have led to the finding of additional infections, but this is by no means certain.

Our observations also showed that parasites are frequently more easily demonstrable in the first passage than in the second or the third passage. For instance, of the 16 infections seen at the first passage, only 9 were patent in second-passage mice. Most likely parasites were present in most of these at subpatent levels since, in most instances in which an attempt was made to maintain strains for study, continuation of passaging resulted in the recovery of strains which became progressively more virulent to mice. On the other hand, in a few instances strains positively identified morphologically in the first passage failed to grow out in subsequent passages.

Comparison of intraperitoneal inoculation with and without cortisone. Having determined that multiple passaging did not greatly increase the number of infections demonstrated, a comparison was made to determine if the use of cortisone would result in the detection of additional infections (procedures 1 and 2). Altogether, 58 trials were made in which identical procedures were followed except that one group of mice received no cortisone while the other received 5 mg per mouse of cortisone acetate intramuscularly at the time of inoculation.

The results are shown in Table 1 (first two columns). Of the 24 infections demonstrated, 22 were seen after the use of cortisone; the same number was seen in the groups in which cortisone was not used. Thus, each method resulted in the detection of the same number of infections and each method disclosed two infections not shown by the other.

The use of cortisone as administered in this experiment did not increase the number of infections found. Even so, it was noted in our work that more mice actually died of toxoplasmosis or died earlier in the cortisone groups as compared with the corresponding noncortisone groups. On the other hand, death from infections other than toxoplasmosis was much more frequent in the cortisone-treated mice, probably due to increased susceptibility to bacterial infections.

In both procedures mice were sacrificed and examined after 1 and 2 weeks, but this could not be done with perfect regularity because some of the mice died of toxoplasmosis or from other causes. Analysis of the results showed that in at least five instances mice from groups in which no parasites were found at 1 week, had demonstrable parasites after 2. Conversely, there were at least four instances in which parasites were demonstrable after 1 week but not seen in the same group after 2. Most likely these results were related to the intensity of the infection in the donor cat. The effect of this on the rate of development of the infection emphasizes the desirability of making examinations at both periods.

TABLE 1

Comparison of the number of infections demonstrated by intraperitoneal inoculation with and without cortisone and by intracerebral inoculation

	I.p. inoculation		I. c. inoculation without cortisone
	With cortisone	Without cortisone	
Cats tested	58	58	58
Infections demonstrated	22	22	12
Demonstrated by this method alone	2	2	0

Comparison of intraperitoneal and intracerebral inoculation (procedures 2 and 4). The results of this comparison are shown in the last two columns of Table 1. Only about half the infections detected by the i.p. method were demonstrated also by the i.c. method. If comparison be extended to the other methods summarized in Table 1, it can be seen that in each case in which recovery was made intracerebrally, *all* of the other methods also revealed parasites.

This indicates that there is no advantage to the use of the i.c. method. Undoubtedly the lack of sensitivity of the i.c. method was due to the very small inoculum which may be injected i.c. compared with the large amount which may be given i.p.

Comparison of intraperitoneal method followed by serological study, with same method followed by sacrifice of mice at 1 and 2 weeks (procedures 5 and 3). The i.p. inoculations of the previous experiments were followed by the examination of sacrificed mice at 1 or 2 weeks after inoculation. Since the majority of strains newly isolated from

TABLE 2

Comparison of intraperitoneal method followed by sacrifice at 1 and 2 weeks with same method followed by serological study at 6 to 8 weeks

	Sacrifice at 1 to 2 weeks	Dye test at 6 to 8 weeks
No. of cats	56	56
No. in which parasites were seen	22	22†
No. in which recipient mice were dye-test positive	—*	20
Positive this method alone	4	2

* The mice sacrificed at 1 and 2 weeks were not dye tested.

† Parasites were found either in brain smears from first-passage mice or in mice of subsequent passages.

the cat, and probably from other hosts, do not kill the first-passage mice, it is possible to hold these mice indefinitely. Advantage of this has been taken by Aagaard and others, who have used the dye test as an indicator of infection in mice held for sufficient time for the dye-test reaction to become positive.

We have compared the results from this procedure with results obtained by parasitological examination after 1 and 2 weeks (Table 2). Both methods revealed most of the infections and each revealed a few not proved by the other. The discrepant findings most likely occurred in cases in which there were few parasites in the inocula with the result that only a part of the mice received infective material.

In most instances the dye tests of the mice were positive at a level of 1:1,024 or higher. We considered this a reliable indication of infection, as uninoculated mice have so far in our experience proved uniformly dye-test negative. To eliminate the possibility that the dye-test finding could have been due to a reaction to dead parasites, we examined smears from the brain of most of the dye-tested mice. In 95 per cent of the examinations the results of the dye test could be confirmed parasitologically by the finding of pseudocysts. In this work examination was made both of fresh suspension and of smears made by crushing bits of brain tissue. It was found that pseudocysts were readily recognizable in fresh preparations and that the examination of fresh material was more efficient than the examination of stained preparations.

Per os infection (*procedure 6*). The hypothesis that natural *Toxoplasma* infection may be acquired through the ingestion of infected tissues has received considerable attention recently. Even so, there is not complete unanimity of opinion that p.o. infection can be regularly achieved. The greatest weight of evidence indicates that pseudocysts readily give rise to infection on ingestion but that proliferative forms do not.

In our experiments we compared groups of mice injected intraperitoneally as usual (procedure 2) with other groups fed brain tissue from the same cat. The tissue was fed as chunks of brain and most of the mice ingested a gram or more as compared with the much smaller amount injected (original tissue volume of about 0.25 g). In all, 25 comparisons were made. Fourteen infections were demonstrated by i.p. inoculation as compared with only five by feeding. The p.o. method was much less efficient than the parenteral, but it is of interest that we have been able to maintain two of the strains recovered by feeding through three to five p.o. passages in mice and there seems to be no reason why they cannot be maintained indefinitely in this manner.

Comparison of intraperitoneal inoculation of mice and rabbits (*procedures 2 and 7*). In a few instances comparisons were made of the results from the i.p. inoculation of rabbits and mice. The purpose was to see if advantage could be taken of the fact that much larger inocula could be used in the rabbit.

Eighteen comparisons were made and in 13 instances neither rabbits nor mice gave evidence of infection. In one instance parasites were demonstrated in mice but not in rabbits. In one instance no parasites were demonstrated in mice but the dye test of one of the group of two rabbits rose from negative to 1:4,096. The dye test of the other rabbit remained negative.

In three instances parasites were found in mice and the dye-test titer of at least one of the two rabbits rose from negative to 1:1,024 or higher. In none of the instances were parasites seen in smears from the rabbits and efforts were, therefore, not made to continue passage of the parasites presumed to be present on the basis of the changed serological reaction.

The marked serological response would cer-

tainly indicate that the rabbits had become infected with *Toxoplasma*, but the rabbits became infected no more consistently than the mice. On the basis of the results presented, the use of rabbits would appear to have no advantage, but the experiments were insufficient in number to warrant a firm conclusion.

DISCUSSION

The principal purpose of the research outlined was achieved; that is, a technique for routine use in epidemiological and diagnostic studies was devised. This technique, which we have followed extensively in studies in our laboratory, is as follows:

Each of four or more mice are inoculated intraperitoneally with one ml of 1:4 tissue suspensions (usually brain) from the suspected host. One mouse is sacrificed at 1 week and another at 2 weeks and all mice dying are also examined. If infection has not been demonstrated, the remaining mice are held until 6 or 8 weeks then bled from the orbital sinus for a dye test. If the dye test is strongly positive, infection is considered extremely likely and is usually confirmed by the finding of pseudocysts in brain smears.

The above technique permits early diagnosis in cases in which parasites are evident when mice are sacrificed early. Infections not evident early are then detected after the serological reaction of the mice becomes positive. Diagnosis based on the serological response of the mice followed by finding of pseudocysts has the advantage of not depending solely upon morphological criteria. Thus, the possibility of confusion of *Toxoplasma* with a morphologically similar organism is minimized.

The infection *per os*, while much less efficient than the parenteral method, does demonstrate that infection may occur in this manner from the consumption of infected tissues from naturally infected hosts. The tissue used in the present study was brain, and similar work with infected muscle of naturally infected hosts should be attempted.

The use of rabbits did not result in increased recoveries in spite of the fact that much larger inocula may be used than in mice. Since rabbits are often found infected spontaneously, there would appear to be no advantage to the use of this animal.

SUMMARY

Comparisons of different techniques for the isolation of *Toxoplasma* from naturally infected hosts were made with the following findings:

1. The use of multiple-animal passages did not result in the recovery of a significantly larger number of infections.

2. The use of cortisone in the dosages employed in the present work did not result in increased recovery rates.

3. Intraperitoneal inoculation was found to be much more efficient than intracerebral inoculation, possibly due to the much larger inocula which could be employed.

4. Intraperitoneal inoculation followed by sacrifice of inoculated mice at 1 and 2 weeks was about as efficient as intraperitoneal inoculation of mice followed by serological testing and examination at 6 to 8 weeks.

5. Infection *per os* was much less efficient than intraperitoneal inoculation, but was successful on a number of occasions.

6. Rabbits inoculated intraperitoneally became infected no more consistently than mice in spite of much larger inocula, thus indicating no advantage in the use of this animal.

7. An efficient technique for routine use was devised.

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THE ISOLATION AND IDENTIFICATION OF TURLOCK VIRUS IN TISSUE CULTURE[1]

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During the course of surveillance studies concerned with the prevalence of Western equine encephalomyelitis (WEE) and St. Louis encephalitis (SLE) virus infections in *Culex tarsalis*, the most important known vector of these viruses in California, isolations of an unknown viral agent were repeatedly made in mice and in embryonated eggs. This agent, designated as the Turlock virus has been described *in extenso* elsewhere (Lennette *et al.*, 1957a, 1957b). The purpose of the present communication is to describe the isolation of the Turlock virus from mosquitoes through the use of avian cell cultures as the susceptible host system and the identification of the virus by means of a colorimetric neutralization test.

MATERIAL AND METHODS

Collection of mosquitoes. Mosquitoes were collected as described elsewhere (Lennette *et al.*, 1956; Welsh *et al.*, 1958). The specimens from each capture-lot were sorted according to species, and lots of 50 female *C. tarsalis* were prepared, deviations from this number occurring only at the beginning and the end of the season when the population densities were low, or in collecting areas where the prevalence of this species was low. The sorted mosquitoes were handled and shipped as described elsewhere (Lennette *et al.*, 1956; Welsh *et al.*, 1958).

Processing of mosquito pools. The mosquito pools were tested immediately upon receipt in the laboratory or within a few days thereafter. The specimens were processed according to the procedure outlined elsewhere (Lennette *et al.*, 1956; Welsh, *et al.*, 1958).

Inoculation of mice and embryonated hens' eggs. Mice and embryonated hens' eggs were inoculated with suitably processed mosquito-pool suspensions; albino Swiss mice of 5 to 6 weeks of age were inoculated intracerebrally with 0.03 ml and 10- to 11-day-old chick embryos were inoculated in the amniotic cavity with 0.1 ml.

Neutralization tests in mice. Freshly isolated strains of the Turlock virus are pathogenic for very young suckling mice and for chick embryos, but not for adult mice. Consequently, neutralization tests were performed in 1- to 3-day-old mice by the combined intraperitoneal-intracerebral routes. The procedure is described in an earlier paper (Lennette *et al.*, 1957a).

Choice of cell type for cell cultures. Reasons for the use of chick-embryo cells for the in vitro isolation of WEE virus from mosquitoes are given elsewhere (Welsh *et al.*, 1958). Since the findings reported here formed a part of this previous study, the same cell-culture system was used throughout.

Preparation of chick-embryo cells and culture medium. This is described in a previous paper, which deals with the isolation of WEE virus from mosquitoes (Welsh *et al.*, 1958).

Containers used for tests. These did not differ from those employed in the earlier work (Welsh *et al.*, 1958).

Procedure for isolation of viruses. Suspensions of trypsinized and washed chick-embryo cells were dispensed into tubes in 0.9 ml amounts by means of a Cornwall automatic syringe. The syringe was supplied from a flask holding the stock cell suspension; during the dispensing operation the suspension was continually agitated by means of a magnetic stirrer. Each mosquito-pool preparation was inoculated into four tubes of cell culture, each tube receiving 0.1 ml of the suspension. In many instances the mosquito-pool suspensions were toxic for the chick-embryo cells as shown by partial or complete destruction of the cell sheet within 24 to 48 hours after inoculation. Insofar as the present observations go, the toxic factor or factors did not *seem* to hamper the survival or growth of the virus, and manifestations of

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toxicity disappeared with subpassages of the material. The following procedure, consequently, was adopted as a routine. Cultures were inoculated with mosquito-pool suspensions and set to incubate at 35–37°C for 5 days; they were not examined microscopically during this period. On the fifth day, the material from each group of four tubes representing a single mosquito pool was harvested and pooled. This was done by scraping any remaining adherent cells off the glass, withdrawing the fluids and suspended cells with a pipette and pooling these materials with materials similarly obtained from other tubes of the group. Each pool of culture material was then inoculated into another set of four freshly prepared tubes, each tube receiving 0.1 ml. The tubes representing the second passage were examined microscopically at 2, 4 and 7 days postinoculation. If one or more tubes of the set of four showed from 2+ to 4+ degeneration at any of these time intervals, the cells and fluids of all four were harvested and pooled and stored in a mechanical refrigerator at −20°C until they could be examined in the identification scheme outlined below. Those tubes which showed no evidence of cellular degeneration at the two earlier examinations (2 and 4 days) were held for a total of 7 days, at which time they were read and either passaged or discarded, depending upon the presence or absence of cytopathic changes. The same procedure was followed in the case of the third-passage material, except that if no cytopathic effect was evident by the seventh postinoculation day, the inoculum was regarded as negative and the cultures were discarded.

Procedure for identification of viral isolates. Colorimetric tests for the assay of poliomyelitis virus and antibody have been described by several workers (Salk *et al.*, 1954; Lipton and Steigman, 1955; Robertson *et al.*, 1955). In all of these methods, falling dilutions of a patient's sera are tested against a known standard test dose of virus. A recent publication (Lennette, Neff and Fox, 1957) from this laboratory describes a colorimetric method for the identification and typing of adenoviruses; and another (Welsh *et al.*, 1958) describes a similar method for the identification of WEE virus. These methods are based on two factors, namely, knowledge as to the expected degree of infectivity of fresh isolates for cell cultures, and the amount of specific immune serum required to protect the cells against the amount of the virus expected to be present in a given dilution of the isolate. The following procedure is based on the same considerations.

The colorimetric test as utilized for the purposes of the present study was performed as follows. Plastic (polystyrene) panels containing 96 depressions, or cups, in 12 rows of 8 each, were used. A 1×10^{-2} dilution of the virus isolate was made in growth medium and delivered in 0.2-ml amounts to each of 16 cups on the plate. To one set of four cups was added an equal amount of normal rabbit serum diluted 1:32 in growth medium. To the second set of four cups were added 0.2-ml amounts of WEE immune serum (rabbit) diluted 1:32, and to the third set of four cups were added 0.2-ml amounts of SLE immune serum (rabbit) also diluted 1:32. To the fourth set of four cups were added 0.2-ml amounts of Turlock virus immune serum (hamster) diluted 1:8. (All sera were inactivated at 56°C for 30 minutes prior to use.) The serum-virus mixtures were incubated for 30 minutes at room temperature, and then 0.2-ml amounts of chick embryo cell suspension were added to each cup. Finally, 0.7 ml of mineral oil was added to each cup to provide a seal. The entire plate was then covered with a sheet of sterile aluminum foil, stacked with other plates and incubated at 35–37°C for 5 to 7 days. The tests were read when the cell controls, consisting of 0.2-ml amounts of cell suspension added to 0.4-ml amounts of diluent, had reached a pH of 6.6 to 6.8. A pH reading of 7.4 to 8.0 was taken as evidence of cell death, i.e., viral activity, and a pH reading of 6.8 to 7.2 as evidence of cell growth, i.e., neutralization of viral activity.

EXPERIMENTAL

At the very beginning of our studies on the application of tissue-culture methods to the isolation of viral agents from mosquitoes, cytopathic effects were encountered in a considerable proportion of the first-culture passages of mosquito-pool suspensions. Subculturing through the second and third passage levels showed that in many cases (about 30%—see further below) the cytopathic effect was due to toxic factors. In the remaining instances, cell destruction appeared referable to the activity of a

viral agent. Colorimetric neutralization tests performed as outlined above and elsewhere (Welsh *et al.*, 1958) showed that in most cases the cytopathic agent could be neutralized by WEE immune serum; in the few remaining instances, the cytopathogenic activity of the agent could not be prevented by WEE serum or SLE serum alone, or by a mixture of the two. The cytopathogenic activity was therefore regarded as quite probably due to the Turlock virus, an agent isolated in this laboratory on numerous occasions from *C. tarsalis* and other mosquitoes by inoculation into embryonated eggs.

In order to facilitate identification of viruses recovered in the type of cell-culture system used here, it was considered desirable to have a colorimetric technic comparable to that previously described in connection with the WEE virus (Welsh *et al.*, 1957), so that a uniform method employing a variety of immune sera could be utilized. Application of a colorimetric method to the determination of cell death or cell survival, i.e., the presence or absence of neutralization, requires information on two major points, namely the concentration of virus generally present in a fresh isolate and the concentration of specific immune serum necessary to neutralize completely the infectivity of the isolate for the cell culture. Such information can be obtained by block titrations in which falling dilutions of serum are tested against falling serial dilutions of virus (Welsh *et al.*, 1958). Thus, in the case of WEE and SLE virus isolates, it was found that the general level of virus present could be completely neutralized by a 1:32 dilution of the immune serum that was employed and that a virus dilution of 1×10^{-2} could be used in the identification scheme without fear of missing the infectivity end-point.

A similar scheme was worked out in the case of the Turlock virus and is illustrated in Table 1. The data in Table 1 show that the stock virus strain used had a $TCID_{50}$ titer of $1 \times 10^{-5.0}$, and that this amount of virus could be completely neutralized by a 1:8 or a 1:16 dilution of the specific immune serum, but only partially by a 1:32 or a 1:64 dilution, and not at all by greater dilutions of the serum. Quantitatively, a 1:8 or 1:16 dilution of immune serum was capable of neutralizing 5 or more full logs

TABLE 1

Block assay to determine neutralizing capacity of Turlock-immune serum against Turlock virus

Serum		Dilution of virus*							$TCID_{50}$ endpoint titer of virus
Type	Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
Turlock-immune serum S240 (rabbit)	1:8	+	+	+	+	+	+		$<10^{-1.0}$
		+	+	+	+	+	+	+	
	1:16	+	+	+	+	+	+		$<10^{-1.0}$
		+	+	+	+	+	+	+	
	1:32	−	−	+	+	+	+		$10^{-2.5}$
		−	−	+	+	+	+	+	
	1:64	−	−	−	+	+	+		$10^{-3.5}$
		−	−	−	+	+	+	+	
	1:128	−	−	−	−	+	+		$10^{-5.0}$
		−	−	−	−	+	+	+	
Normal serum (rabbit)	1:8	−	−	−	−	+	+		$10^{-5.0}$
		−	−	−	−	+	+	+	
No serum	—	−	−	−	−	+	+	+	$10^{-4.5}$
		−	−	−	−	+	+	+	

* + = cell growth—final pH 6.8–7.2 (no viral activity); − = cell death—final pH 7.4–8.0 (viral activity). Each + or − represents one cup on the plastic plate used in the test.

of virus, a 1:32 dilution could neutralize 2.5 logs of virus and a 1:64 dilution could neutralize 1.5 logs of the virus; at 1:128 the serum was diluted beyond any demonstrable neutralizing activity. With respect to the concentration of virus present in fresh isolates, this was uniformly found to be sufficiently high to permit the use of a 1×10^{-2} dilution of the isolate in the identification test, just as had previously been found true in the case of WEE virus isolates (Welsh *et al.*, 1958).

Table 2 illustrates the range of infectivities encountered in Turlock virus isolates and the uniform capacity of a 1:8 dilution of Turlock-immune serum to neutralize maximal amounts of the virus. It will be noted that the isolates shown in Table 2 range in infectivity from $1 \times 10^{-3.5}$ to $1 \times 10^{-6.5}$ or greater and that a 1×10^{-2} dilution of the viral isolate represented from 32 to 32,000 $TCID_{50}$ of virus. As is shown in Table 2, a 1:8 dilution of the standard Turlock-immune serum used was adequate to neutralize the entire range of infectivities encountered. Consequently, in the routine iden-

TABLE 2

Results obtained in testing unknown viruses isolated from C. tarsalis *against Turlock-virus antiserum**

Mosquito pool number	Immune serum		Dilution of virus isolate					
	Type	Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
145-6	None	–	–	–	–	+	+	+
			–	–	–	+	+	+
	Turlock	1:8	+	+	+	+	+	+
			+	+	+	+	+	+
145-12	None	–	–	–	–	–	+	+
			–	–	–	–	+	+
	Turlock	1:8	+	+	+	+	+	+
			+	+	+	+	+	+
621-36	None	–	–	–	–	–	–	–
			–	–	–	–	–	–
	Turlock	1:8	+	+	+	+	+	+
			+	+	+	+	+	+

* See footnote to Table 1.

tification scheme a 1×10^{-2} dilution of viral isolate was tested against a 1:8 dilution of the standard Turlock-immune serum.

APPLICATION OF TISSUE CULTURE METHODS TO FIELD MATERIALS

Toxicity. In recent years, increasing use has been made of tissue-culture methods for the examination of clinical and field materials of many types. The inherent cytotoxicity of some of these materials has posed problems, and various approaches have been used to circumvent cytotoxic activity. Thus, in the recovery of polioviruses from fecal suspensions, the inoculum has been introduced into monolayer cell cultures and after a suitable interval of incubation to permit viral attachment to the cell, the cells are washed free of the residual inoculum and its cytotoxic components (Schmidt and Lennette, 1957). Such a procedure was not possible in the present work since the inoculum was mixed directly with a suspension of susceptible cells and, as shown in Table 3, approximately one-third of all of the mosquito pools examined proved to be cytotoxic in some degree for chick-embryo cells. Since, as is shown in Table 3, the factors responsible for cytotoxicity could be diluted out on subculture, no attempt was made to modify the processing procedure of the

TABLE 3

Cytotoxic effects of mosquito-pool suspensions on chick-embryo-cell cultures

Passage level	Pools examined	Pools cytotoxic	Per cent of pools cytotoxic
1	294	92	*31.3*
2	294	12	*4.1*
3	222	0	*0*

mosquito pools; subcultivation was utilized as a qualitative criterion as to whether any cytopathic effects encountered were due to the specific activity of a viral agent or were nonspecific (bacterial and mycotic contamination being ruled out).

What effect the early death of cells brought about by cytotoxins may have on the propagation of any virus present in the inoculum is unknown. Comparative studies are desirable to ascertain whether killing of the cells by cytotoxins shortly after the introduction of the inoculum precludes multiplication to demonstrable levels of any virus that might be present, or permits of multiplication only to comparatively low levels.

Recovery of Turlock virus from mosquitoes. While viral reproduction in the cell system is evidenced in a number of ways (e.g., by the appearance of complement-fixing or hemagglutinating antigens) the occurrence of cytopathic effects is the paramount criterion of viral activity; other criteria generally are not employed unless such visual evidence of viral multiplication is lacking. In the present study the appearance of cellular destruction was taken as evidence of viral propagation, cytotoxicity having been ruled out, and no other techniques for detecting the presence of virus in the cultures were used.

During the course of these studies, 545 pools of *C. tarsalis* were examined. As has been described elsewhere (Welsh *et al.*, 1958) 66 isolations of WEE virus and 9 isolations of Turlock were made. Table 4 shows the differences between these two viruses in chick-embryo cultures with respect to the time required by each to destroy 75 per cent to 100 per cent of the cell population (this is usually referred to as 3+ or 4+ cytopathogenesis or degeneration).

It will be noted in Table 4 that all 66 strains (100%) of the WEE virus were isolated by the second-passage level and that subculturing of

TABLE 4

Comparison of the time required by Turlock and WEE viruses to destroy 75–100 per cent of cells in chick-cell culture system

Passage level	Time required to destroy 75–100 per cent of culture cells by mosquito pools containing				
	Hours after inoculation	WEE virus		Turlock virus	
		Pools	Per cent	Pools	Per cent
2	48	61	92.4	0	—
	96	4	6.0	1	11.2
	168	1	1.6	4	44.4
3	48	0	—	0	—
	96	0	—	4	44.4
	168	0	—	0	—
Total isolates		66	100.0	9	100.0

the negative materials through a third passage did not result in any additional isolations. In the case of the Turlock virus, only five of the nine strains that were isolated were recovered by the second-passage level, and the remaining four isolations were made at the third-passage level; subcultivation of negative materials into the fourth cell-culture passage did not result in any additional isolations. Similarly, evidence of viral growth, i.e., cytopathogenic effects, was much more rapidly produced by the WEE virus than by the Turlock virus. Thus, at the second-passage level, in 92 per cent of the cases the WEE virus had produced virtually complete destruction of the cell population within 48 hours after inoculation, whereas none of the strains of Turlock virus had produced any evidence of cytopathogenesis at this time. On the other hand, four of the five (80%) strains of Turlock virus achieved almost complete cellular destruction only within 7 days after inoculation.

Comparison of tissue-culture system with mice and embryonated eggs as host systems. The data in Table 4 indicate that the slow growth of the Turlock virus necessitates at least three blind passages in chick-embryo tissue-culture systems to insure isolation of the agent. It is not impossible that more than three passages would result in a greater number of isolates. However, in the present series a total of 222 mosquito-pool suspensions was cultured through three passages in the chick-embryo cells, and the sensitivity of this procedure for the recovery of Turlock virus strains was compared with the sensitivity of the usual procedure in which embryonated eggs and adult mice are used as the test hosts (Lennette *et al.*, 1957a). These findings are summated in Table 5.

Table 5 shows that in a series of parallel examinations of five mosquito collections totaling 222 individual mosquito pools, the Turlock virus was isolated on ten occasions using embryonated eggs or adult mice as the hosts, and in nine instances using the tissue-culture system, an apparent agreement of 90 per cent.

Table 6 gives a less detailed but more specific comparison of the isolations made in tissue cultures and those made in embryonated eggs and mice. It will be noted first of all that from

TABLE 5

Comparative sensitivity of mouse-embryonated-egg and chick-embryo-cell culture systems for the isolation of Turlock virus

Mosquito-pool collection number	Pools tested in parallel	Strains of Turlock virus isolated in	
		Eggs and mice	Chick-cell cultures (3 passages)
145	44	4	3*
158	47	1	1
159	37	3	3
160	47	2	1
621	47	0	1
Totals	222	10	9

* 1 pool positive in cell cultures, negative in eggs and mice.

TABLE 6

*Further comparison of sensitivity of mouse-embryonated-egg and chick-embryo-cell culture systems for the isolation of Turlock virus**

Virus isolation attempts in mice and embryonated eggs	Virus isolation attempts in chick-cell cultures		Total isolates
	Positive	Negative	
Positive	7	3	10
Negative	2	0	2
Total isolates	9	3	12

* Comparison based on 222 mosquito pools examined in parallel. Cf. Table 5.

the 222 mosquito pools examined in parallel by the tissue-culture system and by the mouse-embryonated-egg system, 12 strains of Turlock virus were isolated. Of the 12 strains, ten (83%) were recovered in eggs or mice and nine (75%) in chick-embryo-cell cultures. Of the ten mosquito pools which yielded a Turlock virus in mice and eggs, seven (70%) also yielded a virus in tissue culture. Similarly, of the nine pools which yielded a virus in the tissue-culture systems, seven (78%) also yielded a virus in embryonated eggs or in mice. From these data, admittedly small, it appears that the two systems, viz., mouse-embryonated-egg and chick-embryo-cell culture, are essentially equal in susceptibility to infection with these virus. These data also suggest, but do not prove, that cytotoxicity of mosquito-pool suspensions did not interfere with the recovery of the virus that may have been present in the suspensions.

DISCUSSION

Studies in this laboratory over the past several years have shown that three viruses, namely WEE virus, SLE virus and the recently described Turlock virus, are present in *Culex tarsalis* and other mosquitoes in the Central Valley of California. The prevalence of infection with these viruses in the mosquito population varies from season to season, and the extent to which mosquito population densities, mosquito infection rates, and the occurrence of human and animal cases of disease are related, remains to be definitively resolved. This is true primarily of the WEE and SLE viruses, whose pathogenicity for man, the Equidae, and certain wild animals is well established. What role the Turlock virus plays in the causation of human or animal disease is unknown; nor has it as yet been established whether *C. tarsalis* is a true vector of the Turlock virus, or whether its presence in this species is merely adventitious and of no significance. The classic methods for the isolation and identification of these viruses, and for studies such as those just highlighted, utilize embryonated hens' eggs and adult (or baby) mice. These are effective and sensitive methods whose continued use will not be affected to any major degree, under most circumstances, by the results presented in this communication or in a previous one (Welsh *et al.*, 1958). In some situations, however, such as those in which mice or embryonated eggs are either unavailable or available in limited quantities because of economic or other factors, the tissue-culture approach offers a sensitive method for the isolation of WEE and Turlock viruses through the use of minimal amounts of avian tissue and a colorimetric method of identification based on the same considerations.

The relative insusceptibility of chick-embryo tissue to the SLE virus militates against its use for purposes of virus isolations from field materials. It appears that in this respect hamster-kidney-cell cultures (Kissling, 1957) may prove preferable, since they have been reported to support the growth of the WEE and Eastern equine encephalomyelitis viruses as well as the St. Louis virus (Kissling, 1957).

SUMMARY

The studies reported here show that the Turlock virus can be isolated in a culture system which utilizes trypsinized chick-embryo cells as the substrate for viral growth. Viral strains isolated by this technique can be rapidly identified in a colorimetric neutralization test based on the use of chick-embryo cells, a single dilution of the viral isolate and a single dilution of a pretested standard immune serum.

A series of 222 mosquito-pool suspensions was tested in parallel in mice and embryonated eggs and in chick-embryo-cell cultures; 12 strains of Turlock virus were recovered. The isolation results suggest that the tissue-culture method used was comparable in sensitivity to the mouse-embryonated-egg host system.

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SELECTIVE FEEDING HABITS OF ANOPHELINES AMONGST AFRICANS OF DIFFERENT AGES

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It has been demonstrated in the West Indies that three species of Caribbean anopheline mosquitoes prefer to feed upon adults, and particularly on men seated outdoors, the biting frequency being related directly to the age of the victim (Muirhead-Thomson, 1951; Muirhead-Thomson and Mercier, 1952). This observation has been confirmed for *Anopheles gambiae* in West Africa where Thomas (1951) collecting by hand, found that when two or three people slept in a hut random mosquito feeding took place, but that when families of six or more were together an attraction towards the adults was apparent. These mosquito feeding preferences have however been queried on theoretical grounds. It is considered often impossible to explain sporozoite rates except on the assumption that the principal gametocyte carriers in areas of malarial endemicity, small children, are frequently bitten (Davidson and Draper, 1953).

The observations recorded in this paper concern the selective feeding habits of *Anopheles gambiae* and *funestus* amongst Africans of different ages. The term selective feeding is, in our opinion, more appropriate because under natural conditions the anophelines with which we have to deal may not be able to exert a preference for a particular victim. The work was carried out in villages situated in a district of malarial holoendemicity in Tanganyika, East Africa.

METHOD

Volunteers of different ages, from amongst the native population of the villages included in the investigation, slept in their own or in mosquito-catching huts constructed of mud with open interior design. In these huts the dormant occupants were subject to attack under natural conditions by the malaria vectors *A. gambiae* and *funestus*, which have in this report been grouped together as it was found that they differed little if at all in feeding habits. The method used to determine which of the people sleeping in a hut had been the victim of a particular blood-fed mosquito was that involving correlation of the differential leukocyte count (Clyde and Shute, 1955), thick blood films taken from the people occupying the hut, and from the blood meals of the mosquitoes feeding therein, being stained with a modified Walker (1952) technique.

RESULTS

The observations are limited to consideration of anopheline feeding selectivity when groups of three, four, or five people were asleep in huts. These numbers are the more important under natural living conditions in the district concerned, where the average number of people living in a hut is only 3.3, and the numbers of huts occupied by more than five people are few.

In huts containing three people, four catches, in which 120 blood-fed anophelines were taken, revealed by our method no feeding selectivity for a particular human age group, the proportion of meals taken from adults and from children of different ages being approximately equal.

In huts containing four or five people, the results of 22 catches, in which 575 blood-fed anophelines were taken, are shown in Tables 1 and 2, respectively.

When the data in Table 1 are combined into the human age-groups 0–5, 6–10, 11–15, and adults, it appears that 36, 60, 71, and 105 anophelines fed, respectively; and in Table 2, 66, 60, 79, and 98. The analysis of the feeding selection under natural conditions is complicated; for example, when four people are sleeping in a hut, all may be adults, or one an adult and the remainder small children, and so forth. The mosquito is rarely provided with a range of potential victims of all ages on any single occasion. Even when a certain family is used repeatedly in these tests, it has been unusual to find any similarity in the mosquito feeding patterns. On some occasions adults, on others children, have provided the majority of the blood meals and, sometimes, the mosquito

TABLE 1

Anopheline feeding habits among four Africans sleeping in a hut

Case No., sex and age of victim	Percentage of anophelines taken at each catch and fed on victim*									
	Catch number:									
	1	2	3	4	5	6	7	8	9	10
1. M 65								14		
2. M 55				7	49	18	16			
4. M 50	68	22	45		23					
5. M 50									27	48
8. M 35								14		
10. F 23	0	22	10							
14. M 15				52	21	9	40			
15. M 15						15				
17. M 14			21							
38. M 13									24	24
19. M 13			24				12			
27. M 10	32	22		17	7	58	32			
30. M 8									31	19
31. M 8								0		
32. M 4								72		
33. F 3	0	34		24						
39. M 2									18	9
Total anophs. fed per catch	19	18	29	29	43	34	25	21	33	21

* For example, in Catch no. 1, the 4 people sleeping in the hut were a man aged 50, a woman aged 23, a boy aged 10, and a girl aged 3. No representative of the 11–15 age group was present. Nineteen anophelines fed on these people, 13 (68%) on the man, and 6 (32%) on the boy. The numbers of anophelines feeding on each victim have been tabulated as percentages for convenience of comparison, but it is emphasized that the totals from which these were derived were as small as, in this column, 19. Where the case numbers in Tables 1 and 2 are identical, the same people are indicated.

appears to have fed partially on one and partially on a second victim.

The means of the percentages of anophelines feeding in huts containing four or five people, and calculated from the data recorded in the Tables, are as follows:

Age	4 People	People
0–5	25	24.5
6–10	24	26
11–15	24	26
Adult	27	23.5

TABLE 2

Anopheline feeding habits among five Africans sleeping in a hut

Case No., sex and age of victim	Percentage of anophelines taken at each catch and fed on victim											
	Catch number:											
	1	2	3	4	5	6	7	8	9	10	11	12
1. M 65						7	24	30	0	0		
2. M 55				52	6							
3. M 55												0
4. M 50	32	48	38									
6. F 45	11											
7. F 35												22
8. M 35											32	
9. F 25						17	0	33				
10. F 23		0	5									
13. F 18												22
14. M 15				6	6							
16. F 15												11
17. M 14			24									
18. M 14											16	
19. M 13			19									
20. M 13		37										
21. M 13					6							
22. F 13						52	16	15	28	34		
24. M 12			14	12	12							
25. F 11												45
27. M 10	43	15		24	70							
28. M 9											0	
29. F 9									14	8		
31. M 8						17			19	16	5	
32. M 4							50	11	39	42	47	
33. F 3	3	0		6								
35. M 1½	11											
36. F ½						7	10	11				
Total anophs. fed per catch	35	27	21	33	17	29	38	27	36	12	19	9

It may appear from these figures that there was in fact not a random feeding distribution, 25 per cent of the mosquitoes having been recorded as feeding on adults, and 75 per cent on children aged 15 years or less. However, the total number of people subjected to the tests, and shown in the Tables, was 16 adults and 29 children. Thus, 25 per cent of the mosquitoes fed on 35 per cent of the subjects (the adults), and 75 on 65 per cent (the children).

CONCLUSION

It appears that anopheline feeding took place at random among Africans of different ages sleeping 3, 4, or 5 in a hut, although on some

occasions particular individuals were selected repeatedly. In the district in which this investigation was performed, adult human males, customarily sleeping with sparse if any coverings, provided a greater proportion of the meals than did women and their babies, who sleep together enveloped in sheets from head to foot. Whilst the mosquitoes in these huts appear to feed on any person available and exposed, the findings do not imply that there is no deviation in the relatively few huts containing more than six occupants.

SUMMARY

In 26 night catches, 695 blood-fed *Anopheles gambiae* and *funestus* were taken in huts containing 3, 4, or 5 Africans sleeping under natural conditions, in a district in Tanganyika, East Africa, where the average number of inhabitants of a hut is 3.3. By the use of the method of leukocyte correlation of the blood of the human occupants of the hut with the blood in the stomach of the fed mosquito, it appeared that there was no consistent deviation of anophelines to certain human age groups.

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HUMAN AND ANIMAL TRYPANOSOMIASIS IN AFRICA[1]

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Africa is not one place, but many places, and the epidemiology of trypanosomiasis, whether of man or of animals, will vary considerably in detail as one goes a thousand miles one way or a thousand miles the other, or climbs from sea level to 6000 feet above, or surveys the scene in the relatively uniformly warm and moist climate of much of Uganda, or in the many areas where a hot, dry season lasting several months intervenes between the rains.

It has been estimated that the area of Africa occupied by tsetse flies, and so threatened by trypanosomiasis, is almost four million square miles, which is larger than that covered by the U.S.A., and it is not surprising that there should be much variation in climate, in vegetation, and in topography. One thing only is common to all the places where the flies breed and therefore survive, and this is that they all are woodlands, bush or forest and (for there to be woods) rainfall must be sufficient. These places, too, are amongst those customarily described as hot. Probably tsetse flies are not found when the mean annual temperature is less than about 70°F.

The enormity of the problem presented to Africa (and to me about to describe it in a short paper) would have been less, of course, had there been fewer species of trypanosomes and fewer species of tsetse flies to spread them. I have listed the species of trypanosomes in Table 1 and of tsetse flies in Table 2, together with some of their important features.

THE TRYPANOSOMES

Table 1, although erring on the side of dogmatism, is reasonably accurate, but Table 2 requires explanation if it is not to be too misleading. I have included it for one purpose only, which is to emphasize that the different species of tsetse fly show a preference for certain types of woodland. This preference has an important bearing on the epidemiology of the two kinds of human trypanosomiasis, and of animal trypanosomiasis. I will return to the point later, but it is emphasised also by the species I have omitted from the Table. These belong mostly to the fusca group: *G. nigrofusca*, *G. medicorum*, *G. tabaniformis* etc., species which tend to be confined to evergreen forest and so to come into contact with man or his domestic animals infrequently. The chief fault with Table 2, of course, is that it is oversimplified. For example, the so-called thicket species are not confined to thickets. It seems true that they require thickets to complete their life history, but the thickets can be small and scattered in more open woodland or even open country over which the flies may roam. The whole subject of the relationship between tsetse flies and vegetation is very complex, and for an introduction to the literature and a summary of the facts I must refer you to Chapter 9 of the late Professor Patrick Buxton's admirable monograph on *The Natural History of Tsetse Flies* (1955a).

Now, before discussing the epidemiology of the trypanosomal diseases, I want to say something about the trypanosomes. Let me begin by reminding you of their relationships and characteristics.

Polymorphism is a feature of *T. brucei*, *T. rhodesiense* and *T. gambiense*; that is to say, three forms of the parasite, long, intermediate, and short, are seen in blood smears taken from natural infections which are, of course, transmitted by tsetse flies. It is characteristic that the polymorphism is eventually lost when trypanosomes of these species are transferred to laboratory hosts such as rats and mice, and maintained by mechanical transfer of blood from host to host. The three species are morphologically identical, and therefore some authorities would prefer to regard *T. rhodesiense* and *T. gambiense* as varieties of *T. brucei*. *T. brucei* itself is not known to infect man, and attempts to establish artificial infections have failed. The two species or varieties infecting man differ in their geographical distribution, in the tsetse which normally spread them, and generally in virulence.

T. gambiense is predominantly West African and very widespread whilst *T. rhodesiense* is

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TABLE 1

African trypanosomes pathogenic in man or domestic animals

Species	Wild hosts	Pathogenic in	Remarks
T. brucei Plimmer & Bradford 1899	African, ungulates	Horses, camels, dogs, sheep, goats & pigs (?)*	Infections also produced in cattle but very rarely severe. Often spontaneous recovery occurs.
T. gambiense Dutton 1902	Not known	Man	Typically a chronic infection lasting months or years and terminating in "sleeping sickness."
T. rhodesiense Stephens & Fantham 1910	Not known	Man (in animals probably similar to *T. brucei*)	Often an acute, virulent infection, fatal within a few weeks or months, sometimes before "sleeping sickness" develops.
T. suis Ochmann 1905	African bush pig and wart hog	Pigs	
T. congolense Broden 1904	African ungulates	Cattle, horses, dogs, sheep, goats.	Typically a chronic wasting infection rarely, if ever, fulminating. Often fatal, but usually only after the infection has lasted some months. Pigs are the least affected of the domestic animals.
T. dimorphon Laveran & Mesnel 1904			A "dimorphic form of *T. congolense*" probably behaving like *T. congolense* but little is known about it except taxonomy.
T. simiae Bruce *et al.*, 1912	African bush pigs & wart hog	Pigs	Fulminating, rapidly fatal infections in pigs. Transient infections in several other domestic animals.
T. vivax Ziemann 1905	African ungulates	Cattle, horses, sheep, goats.	Much variation in pathogenicity with different strains. Some cause little apparent harm; others are severely debilitating and some produce a fulminating fatal infection.
T. uniforme Bruce *et al.*, 1911			A "short form of *T. vivax*" recognized as a distinct species, but little recorded about it except taxonomy.
T. evansi Steel 1885	?	Camels, equines, cattle.	Transmitted mechanically by biting flies, mainly Tabanids. Therefore occurs far from the tsetse belt, as in the Northern Sudan and Egypt.
T. equiperdum Doflein 1901		equines	In Africa, reported only from the North. Remarkable amongst the trypanosomes in being transmitted during coitus.

* There is some uncertainty regarding the pathogenicity of *T. brucei* for pigs. See, *e.g.*, Davey (1948, p. 5); Hornby (1952, p. 6).

TABLE 2

The chief species of Glossina *transmitting human and animal trypanosomiasis in Africa*

Waterside species	Transition-forest species	Woodland-savannah species	Thornbush species	Thicket species
G. palpalis *G. tachinoides*	*G. longipalpis*	*G. morsitans*	*G. longipennis* *G. swynnertoni*	*G. pallidipes* *G. brevipalpis* *G. austeni*

predominantly East African and tends to occur in very scattered and relatively small areas. The distribution has been mapped by Leeson (1953). *T. rhodesiense*, however, has been found in the Belgian Congo (Marneffi, 1955; Evens, 1956) and pockets of *T. gambiense* occur east of Lake Victoria. Infections with *T. rhodesiense* are often acute, although exceptions have been reported; and with *T. gambiense* are typically long drawn out and chronic, although again exceptions have been reported. I shall not attempt any taxonomic argument concerning the exact relationship of *T. rhodesiense* and *T. gambiense*, one to the other and to *T. brucei*, and I will be content to make a simple dogmatic statement. It is this: an infection can be transmitted to man by *Glossina palpalis* and *G. tachinoides*. These are waterside species which frequently come into contact with man, and we call that infection Gambian trypanosomiasis, and say it is caused by *T. gambiense*. An infection can also be transmitted to man by *G. morsitans*, and sometimes *G. pallidipes* and *G. swynnertoni*, *i.e.*, the game-attacking tsetse (species which do not nearly so often come into contact with man) and we call that infection Rhodesian trypanosomiasis, and say it is caused by *T. rhodesiense*. As I will show, the causes of the greatest differences in the epidemiology of the two infections are the habits of the tsetse flies transmitting them.

These three species have another characteristic in common, which is that in the tsetse fly development of the trypanosome occurs in the midgut and salivary glands. *T. suis* shares this feature and, although it is monomorphic, Hoare (1957a, b) classifies it in the *brucei* group.

T. congolense is monomorphic, *T. dimorphon* dimorphic, and *T. simiae* polymorphic, but all three develop in the midgut and proboscis of the tsetse fly and Hoare classifies them together in the *congolense* group.

T. vivax and *T. uniforme* are identical morphologically except in length, and both develop in the proboscis only of the tsetse fly. Undoubtedly, if one accepts *T. uniforme* as a separate species one must admit that many of the records referring to *T. vivax* have unwittingly included *T. uniforme*. Apart from its economic importance *T. vivax* is of interest in three ways. First, it may exhibit extremes of virulence that are very great. For example, I worked in Uganda with a freshly isolated strain which gave such low grade infections in cattle that my experiments were most difficult to interpret (Davey, 1950). A few months later I worked with a strain maintained in the Kenya Veterinary Laboratory by Dr. E. A. Lewis, and that often killed untreated cattle within 2 or 3 weeks. The difference in virulence between *T. gambiense* and *T. rhodesiense* is sometimes as marked in man and in certain laboratory animals such as Indian *rhesus* monkeys.

The second point of interest concerning *T. vivax* is that it has, within relatively recent times, extended its range outside Africa, and is now found in the West Indies, South America and Mauritius. It appears to have been carried to these other countries in cattle imported from Africa and was spread, and continues to be spread, mechanically by blood-sucking flies such as the tabanids. From all the evidence that is available, both direct and indirect, one would think that *T. congolense* is equally capable of being transmitted mechanically. I suppose that this species is not present in South America because it is generally more pathogenic, or at least less often gives rise to a benign infection. As a result, either infected beasts would not have been purchased or they would have died before reaching their new home or shortly afterwards.

The third point is the specificity of *T. vivax*. Most of the species I have listed in Table 1 are readily inoculable into laboratory rodents and monkeys, but *T. vivax* is an outstanding exception. Workers in the West African Institute for Trypanosomiasis Research showed, however, that if inoculation from sheep into rats was accom-

panied within 24 hours by a supplementary injection of a relatively large amount of sheep serum, *T. vivax* could be maintained successfully in its new host (Desowitz, *et al.*, 1952, 1953). After seventy passages the use of sheep serum was unnecessary, and the strain could also be passed into mice. I have had this strain in my laboratory, maintained in mice, for over 4 years, and a few months ago I took its history a step further. I found, much to my disappointment, that it would no longer infect cattle. By contrast, *T. congolense* can be transferred readily into mice from cattle nautrally infected in Africa, and to my knowledge, after at least 12 years continuous passage in mice, readily infect cattle again.

I have included *T. evansi* in the Table because it occurs in Africa. It also occurs, of course, in many other places, and it is not transmitted by tsetse flies, at least not cyclically, and there are no records that it is of importance in Africa except in the lands of the North where the camel is an important beast. Hoare (1957) thinks *T. evansi* arose from *T. brucei* and one would suppose that *T. equinum* at least, and possibly *T. equiperdum*, then came from *T. evansi*. The only evidence, of course, is deductive, and I should point out that even if there is much morphological similarity between *T. brucei* and *T. evansi*, there is also much dissimilarity chemotherapeutically.

TRYPANOSOMIASIS

Following this brief introduction to the trypanosomes, I want to discuss the epidemiology of the diseases caused by them in man and in domestic animals. Again, I must be brief and I must ask you to remember that I can give you only the salient features of the picture.

Human trypanosomiasis. Two bald statements will help to clear the way. First, there is no evidence that any of the African trypanosomes, except *T. rhodesiense* and *T. gambiense*, infect man, which is remarkable when one remembers the ease with which many of the species infect other animals including monkeys. Second, there is no direct evidence that a reservoir of *T. rhodesiense* or *T. gambiense* exists in any wild animal. There is indirect evidence that such a reservoir may exist, particularly for *T. rhodesiense*, when it is also reinforced by strong circumstantial evidence, but there is no direct evidence.

Let us consider first the epidemiology of *T. rhodesiense*. The indirect evidence for a reservoir of this species is exemplified by the work done at the Tinde Laboratory in Tanganyika. Here, for over 18 years, *T. rhodesiense* has been maintained by cyclical transmission in sheep with secondary lines in antelope and monkeys. Periodically its infectivity for man has been checked. The experiment was started by Corson in 1934, and the results are brought up to 1953 by Willett and Fairbairn (1955). Throughout the experiment the infectivity for man has been retained. It would seem, therefore, that in Nature there could be transmission of the trypanosome via the tsetse fly between the game and man. Why then, does it not take place, or at least why should its occurrence be so rare as to make its demonstration difficult? The answer is a combination of several factors. Most of them are concerned with the habits of *G. morsitans*, the chief vector of *T. rhodesiense*. Surprisingly enough to anyone who looks at a map showing only the distribution of *T. rhodesiense*, so scattered and so localised, *G. morsitans* is one of the most widespread of the tsetse; and it is widespread because it can inhabit many types of woodland. It is apparently absent from dense forest, and certainly from open grassland, but between these two extremes there are many kinds of open woods which *G. morsitans* finds favorable. In other words, what is commonly called bush country in Africa is almost synonymous with the haunts of *G. morsitans*. And much of the central belt of Africa is bush country.

The next point to note is that *G. morsitans* is dependent on game for its continued survival. The first positive demonstration of this within recorded history was during the great rinderpest epizootic in 1896 which affected so much of Africa. It killed all sorts of game and cattle alike in enormous numbers, and the subsequent recession of *G. morsitans* was noticed in the northern Transvaal, in the Rhodesias, in Nyasaland, and in Tanganyika. The map of the distribution of *G. morsitans* in Southern Rhodesia compiled by J. K. Chorley (1947) showing the contrast between the area covered in 1860 and the area covered in 1913 is particularly striking. An equally convincing demonstration has since been given, again in Southern Rhodesia, by the results of the much-maligned policy of game slaughter which has been practiced in that country since the early twenties. Anyone who

has experienced the thrill of seeing the game animals of Africa in their natural haunts must deplore this policy, but there is no doubt of its success. The cynic, of course, might observe that it is better for the game to die from a rifle shot than starve as they are driven out of their grounds because freedom for the game and the development of Africa are incompatible ideas. We have it as a duty to posterity to preserve the game, but they can only be preserved in game reserves.

Where *G. morsitans* is present game is present, too. When we have that combination we can take it that trypanosomes which can kill all man's domestic animals are being carried by the tsetse flies. I know of nothing in the whole world of disease quite like this fact of the virtual inevitability of death for unaided cattle, if not for the other domestic animals, in an area inhabited by *G. morsitans*. The African is also aware of the fact, which brings me to the next factor influencing the epidemiology of trypanosomiasis caused by *T. rhodesiense*.

The social system of many of the African tribes is built around cattle, and they do not live where they cannot keep cattle. The *morsitans* areas are, therefore, sparsely populated. In other words, man is not usually part of the *G. morsitans* environment, and the man-fly relation is a tenuous one. Man is bitten almost by accident as it were, which is why *T. rhodesiense* tends to occur sporadically and why only a relatively small number of cases are recorded each year.

I must mention two more factors which also undoubtedly bear on the problem. First, man is not a preferred host for *G. morsitans*. The fly will bite him, but given a choice it would seem to prefer game and cattle, and most authorities think this is even more true of *G. pallidipes*. Second, a person infected with *T. rhodesiense* often becomes ill very quickly and removes himself to his hut, and for much of the time when he could be regarded as a carrier of the disease he has lost his contact with the fly.

What I have said will explain why infection with *T. rhodesiense* is not really an important disease measured by the total number of people who acquire it each year, and it would seem hardly relevant to go back now and discuss what I called the strong circumstantial evidence that a reservoir of this species exists in wild animals. But I think the evidence is of interest, and I think, too, that you are now in a better position to accept it and to believe that a reservoir does exist, even though the disease is far from widespread.

The strongest evidence comes from an experiment in Tanganyika described by Jackson (1955). He was working in an area where there were no human settlements, and where people were forbidden to come except along a few routes. Guards were posted to enforce the regulations. During several years of work 27 cases of trypanosomiasis occurred among the Africans engaged in the experiment. Jackson says: "It is my personal conviction that wild animals must form a reservoir of the disease in Nature, as they have been shown to do experimentally in captivity."

I will close my account of Rhodesian trypanosomiasis by reference to the report for 1955 by Dr. F. I. C. Apted of the Tanganyika Medical Department. You will observe that a reservoir of the disease in game is assumed. The report says that the disease is now largely occupational as the people in the worst areas live in cleared settlements which give protection at home. Infection is contracted when travelling or hunting, fishing, or gathering beeswax. The remark is made that many people now come for blood examination on return to their homes from the tsetse-infested forest. I should, perhaps, add that in 1955, in Tanganyika, there were 923 reported cases of human trypanosomiasis, and the population is greater than 5 million.

The epidemiology of Gambian trypanosomiasis differs from that of the Rhodesian type mostly because the vectors, *G. palpalis* and *G. tachinoides*, will readily bite man even if he is not the preferred host, and because the habits of these flies are such that they can make frequent contact with man. There is also the fact, by no means unimportant, that the benignity of the early stages of the disease means that man can be an efficient reservoir of it. For months on end, even though infected, he may lead an almost normal life, and his goings and comings along the shaded paths that follow the watersides, and across the fords, could bring him often into contact with the fly.

Most of what we know about the epidemiology of the disease could be explained without supposing that a reservoir exists in animals. Where the disease is endemic, as it is, or was, over much of West Africa and in a few small areas of East

Africa, man himself could maintain it. Where an epidemic flares, as it did in the first years of the century in Uganda, man could have introduced the disease.

The epidemic in Uganda, although historical, is worth pausing to consider. As Buxton (*loc. cit.*, p. 658) says: "This epidemic must be regarded as one of the greatest in human history." At that time, of course, there were very few white people in Uganda, and the collection of accurate data must have been extremely difficult. Bell (1906, quoted by Buxton, *loc. cit.*), however, making due allowances, estimated that 200,000 deaths had been caused by the disease in 5 years, and these out of a population of about 300,000. Exactly how the epidemic started one will never know, but it would appear to have been an isolated experience in this part of Africa, and to have been caused by the introduction of the trypanosome into an area where everything else was ready for its transmission.

G. palpalis is very common around Lake Victoria, and on the islands scattered on the lake. This tsetse can exist on many hosts, including crocodiles and other reptiles as well as game, but it will also bite man and one must suppose that before 1901 the African on the lake shores and on the islands was frequently bitten. That he flourished at all where he did must have meant that the tsetse were not infected. How then did *T. gambiense* come into the area; with man from, say, the Belgian Congo, or with game? The evidence that game might be a reservoir of *T. gambiense* is not so good as it was for *T. rhodesiense*. There are several records that the cyclical transmission of the trypanosome is possible through pigs, goats and antelopes, but there are no records of the trypanosome having been recovered from any of the domestic animals or from game, and not even good circumstantial evidence, apparently, that infection might come that way. A reservoir in domestic animals such as pigs could obviously be of considerable importance, but the importance of game will have the same limitations as it had for *T. rhodesiense*. It would be a potential danger always to be remembered; yet in the day to day spread of the disease among human beings it would be negligible. Man cannot live with his domestic animals where there are game and tsetse, and he rarely chooses to live without them. If he does live without cattle he will cultivate. If he cultivates he will clear the bush. In doing so he will destroy the habitat of the game tsetse, and his presence will drive away the game. If his clearing is big enough he can keep cattle and other animals, and his break with the world of the game is then substantially complete.

But he can do all this and still be threatened by *G. palpalis* and *G. tachinoides*, because he can live in juxtaposition with them and come almost daily into contact with them. These tsetse are quite unspecialised in their feeding habits. They can live on many hosts or on man alone. Often they inhabit very restricted areas such as the thick cover of bushes and trees along a waterside. A clearing may extend to within a few yards of the water's edge and still leave a habitat favorable to the tsetse. Sometimes, the tsetse can fly out from its shelter and attack man working in the fields and often, of course, man will go to the water. He may walk in the shade along its edge, or gather wood for his home there, or cross it, or fish, or go to it for supplies. Under many circumstances, over much of West Africa, conditions are such that frequent contacts between man and these tsetse are possible, which is the explanation of why Gambian trypanosomiasis was widespread. And I must stress that *frequent* contact is necessary for the disease to be important (see, *e.g.*, Hutchinson, 1953).

An extreme example, quoted also for its interest, will illustrate how easily the man-fly contact could be made. In parts of Northern Nigeria "people lived in considerable townships surrounded by a wall with gates. Under British rule the defences crumpled into ruin and the moat outside the wall became dense thicket in which *G. tachinoides* bred, the insects flying over the old wall into the town to feed on man or domestic animals. In such a case one may suppose that trypanosomiasis was frequently contracted in the town or village, perhaps even inside the house" (Buxton, *loc. cit.*, p. 676).

The control of human trypanosomiasis. I have said enough to show that for both Gambian and Rhodesian trypanosomiasis man himself is the important reservoir of the trypanosomes. Even if game is infected, man will acquire the organisms from that source only rarely. Good drugs then, will do a great deal to reduce the incidence of the disease and, used widely and well, could make the disease relatively rare. That is not to say the task is easy. Mass diagnosis and mass treatment, both

of which are necessary, are difficult tasks to organize in a country as backward as much of Africa is. But they have been carried out on a very large scale during the last 15 or 20 years in all the territories where the disease was important, and the results are impressive (see, *e.g.*, Gall, 1954; and the special number of Médicine Tropicale, 1953). The incidence has literally tumbled, and today it is accurate to say that human trypanosomiasis in Africa is no longer a major disease and its importance grows less and less. During 1956, in Northern Nigeria, (Nor. Nig. Med. Dep't., 1956) for example, an incidence of only 0.15 per cent was found amongst the almost two million people who were examined in the endemic areas. There is still room for improvement in the drugs, particularly in those used for the treatment of the cases exhibiting involvement of the nervous system, but even a perfect drug would now have only local importance.

I must not give the impression, however, that chemotherapy has achieved all that has been done for the control of Gambian and Rhodesian trypanosomiasis, particularly the Gambian type. I would be doing a grave injustice to the work of entomologists such as K. R. S. Morris of the Gold Coast, and T. A. M. Nash of Nigeria, and their predecessors like Ll. Lloyd and W. B. Johnson, were I to suggest this. They and their work, without resort to drugs or insecticides, have made whole areas safe for human beings. Their studies, and others like them, of how to make a habitat of *G. palpalis* or *G. tachinoides* uninhabitable for the flies with the minimum expenditure of labor, make fascinating reading, (see, *e.g.*, the summary by Buxton, 1955 b). What the entomologist can achieve when he is aided by good administration, and when sociology is studied as well as entomology, is epitomised by what is called the "Anchau Rural Development and Resettlement Scheme" (Nash, 1948). Anchau is in Northern Nigeria and, in 1934, about a third of the inhabitants of the area were suffering from trypanosomiasis. Both *G. palpalis* and *G. tachinoides* were present and mostly confined to the vegetation along the banks of the streams running through the area. It was decided to eliminate both species by clearing the trees and bushes and, wherever possible, clearing them discriminatively. Finally, an area of 712 square miles was brought under control. When I had the privilege of visiting there in 1950 it contained flourishing communities totalling more than 60,000 people integrated into a way of life that seemed to me to contain everything that was best in the African system, and to point to what could be the future.

Trypanosomiasis in domestic animals. Some of my remarks concerning the epidemiology of human trypanosomiasis will have allowed you to imagine the problem of the disease as it affects the domestic animals. The game carry trypanosomes which can infect them all and cause grievous disease, particularly in cattle. Cattle are very important in the social life of many of the Africans and could be tremendously important in the economic development of their country. All the tsetse appear to feed on cattle as readily as they do on game. Probably all can carry the pathogenic trypanosomes even if not with equal facility, but some, of course, due to their habits, are more important vectors than others. Most authorities would put the far-ranging *G. morsitans* at the top of the list, and *G. pallidipes* next, but *G. swynnertoni*, *G. palpalis* and the others have local importance, and I must add that the term "local" may cover a very large area indeed.

The problem, then, is simple to enunciate. It is this: where there are game and tsetse, cattle cannot live, and there is no easy way of controlling the tsetse because the area involved is so vast. Discriminative or selective clearing of the forest and bush will kill some species in some climates, and ruthless clearing will kill them all. The modern insecticides, too, will kill them if contact between fly and chemical can be achieved. I have already remarked that slaughter of the game will cause *G. morsitans* to disappear. But all these measures are very costly and very difficult to apply over the enormous areas involved. All of them are being used, the emphasis being put on one or other depending on local circumstances. The point I want to make clear, however, is that there is no foreseeable quick solution to the problem. One cannot say, or even hope, that Africa will be free of tsetse fly in 10 years, or 20 years, or much longer, for that matter.

How, then, will the problem be solved? There is a phrase, "the inevitability of gradualness" which gives the answer, I think. The implications are patience, although I hope not too much, and attack. Always attack with a controlled push forward of people and livestock, farms and

ranches, into the tsetse areas. Within memory the reverse has taken place in many parts of Africa and tsetse and game have taken over lands once occupied by people with cattle. Surely, the wheel can be turned again. An example will show what I mean.

In Ankole, in the west of Uganda, tsetse has been advancing for years, pressing from the south where Uganda runs into Tanganyika. It is known that in the second half of the last century, when white explorers were first penetrating into that country, flourishing communities of cattle owners lived where now there are tsetse (Ford and Hall, 1947). The advance of the tsetse followed a series of disasters that came to the people. Raids from other tribes, rinderpest, smallpox and other diseases all played their part. The hold of the people on their land was gradually lost, and the bush grew up and the tsetse came. It seems that a slow retreat of people and cattle has been continuing in that area ever since. About 3 years ago, however, the Veterinary Department in Uganda called an about-turn and they commenced to put cattle and people back into tsetse country to the north of Mbarara.

The cattle are being protected from trypanosomiasis with a drug called Antrycide. Will the presence of people and cattle, with the guidance of veterinary officers, entomologists and agriculturists, make the land unacceptable to tsetse? There is every chance that it will.

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AN EVALUATION OF THE SKIN SCARIFICATION TECHNIC FOR THE RECOVERY OF MICROFILARIAE OF *ONCHOCERCA VOLVULUS*

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With the introduction of the skin-scarification smear (Mazzotti, 1951) an important new technic was added for the diagnosis of *Onchocerca volvulus* infections. Nevertheless, there is relatively little precise information on a number of factors which must be considered in carrying out and interpreting this diagnostic procedure. These include the anatomical region chosen for the smear, the number of smears needed to give significant results, the variations in different smears taken simultaneously from the same anatomical region, variations in smears repeated on different days from contiguous sites and, finally, the absolute efficiency of this technic and its efficiency as compared with the skin biopsy technic.

This paper reports the results of three studies made in an attempt to provide answers to some of the questions raised above. No attempt is made to determine accurately the parasite locations and their relation to distributions of microfilariae in the skin. It is hoped that more conclusive data on this point can be made available later.

MATERIALS AND METHODS

The cases of onchocerciasis studied were from the treatment clinic of the Liberian Institute and the labor force of the Firestone Plantations Company.[1] Only adults were studied. They included both males and females and their infections ranged in density from light to heavy. It was not possible to determine the duration of infection in any of the subjects.

The skin-scarification technic consisted of cutting with a razor blade five superficial linear incisions about 1 mm apart and 1 to 2 mm long. Blood and tissue juices were expressed from the incisions by manual pressure, collected on a slide as a thick smear and stained with Giemsa stain. When necessary, smears were decolorized and restained with hematoxylin. Smears were searched for microfilariae with the 16-mm objective of the microscope and diagnosis was confirmed with the oil-immersion lens.

[1] The co-operation and assistance of the Firestone Plantations Company Management and Health Services are acknowledged with thanks.

EXPERIMENT 1

This experiment was designed to provide comparative data on the numbers of microfilariae in the different superficial anatomical regions of the body as shown by skin-scarification smears.

One hundred and twenty-five subjects, selected on the basis of positive scarification smears, were studied. Two skin-scarification smears were made, one from each side of the body, at the following seven anatomical regions: (1) temple, near outer canthus; (2) shoulder, center of scapula; (3) hip, below greater trochanter; (4) calf, outer lateral surface; (5) ankle; (6) forearm, outer lateral surface; (7) wrist, outer lateral surface.

Results. Detailed results are presented in Table 1. They demonstrate that the rate of recovery of microfilariae is related to the site of examination, the lower limbs and hips showing a much higher concentration of parasites than the upper part of the body or arms. Further, smears from the hips gave both the highest parasite recovery rate (85%) as well as the greatest number of microfilariae per smear. By combining the results of the four scarification smears from the hips and calves, 98% of the positives could be diagnosed.

EXPERIMENT 2

It was the purpose of this experiment to investigate variations in the numbers of microfilariae recovered in smears taken simultaneously from different sites in the same anatomical region.

Eight infected subjects were examined for microfilariae at 16 regions of the body. Smears were made from six different sites within the same anatomical region, three from the left and three from the right side. Thus, a total of 96 smears were examined from each subject. Further, as examination sites included flexor and extensor surfaces, it was possible to compare microfilariae-recovery rates from these two surfaces. The following 16 regions were studied: (1) temples, near outer canthus; (2) shoulders

TABLE 1

The occurrence and density of Onchocerca volvulus *microfilariae in skin-scarification smears taken from the right and left side at seven different body levels of 125 infected subjects*

Site	Positives		Positive right side		Positive left side		Total microfilariae	Microfilariae per pos. smear
	No.	%	No.	%	No.	%		
Hip	106	85	98	78	93	74	1177	6.3
Calf	98	78	74	59	79	63	622	4.1
Ankle	93	74	83	66	86	69	1025	6.1
Shoulder	70	56	50	40	50	40	229	2.3
Arm	27	22	18	14	21	17	73	1.9
Wrist	24	19	16	13	14	11	58	1.9
Temple	7	6	6	5	4	3	13	1.3

(extensor) and chest (flexor); (3) umbilicus region (flexor) and lumbar region (extensor); (4) hips, near greater trochanter; (5) thighs, mid-flexor and -extensor surfaces; (6) legs, mid-flexor and -extensor surfaces; (7) ankles, flexor and extensor surfaces; (8) forearms, mid-flexor and -extensor surfaces; (9) wrists, flexor and extensor surfaces.

Results. The eight subjects showed wide variations in the intensity of their infections. The infection of lowest intensity showed microfilariae in but three of the 16 anatomical regions examined. Of the 18 smears taken from these three regions only five were positive: two from the hip, one from the flexor surface of the thigh and two from the extensor surface of the calf. In each positive smear only a single microfilaria was seen. Skin-scarification-smear findings in two representative subjects, one with a relatively high and the other with an infection of low intensity are shown in Tables 2 and 3, respectively. A study of Table 2 shows that microfilariae were recovered in at least one of the six sites of all anatomical regions and, in the regions of the hip, thigh and calf, all smears were positive. It can also be seen that wide variations occurred in numbers of microfilariae recovered from different sites within the same anatomical region with counts ranging from 0 to 24 in the forearm, 0 to 15 in the wrist, 2 to 55 in the hip, 4 to 33 in the thigh, 16 to 89 in the calf and 0 to 13 in the ankle. In general, wide variations in numbers of microfilariae recovered from different sites in the same anatomical region characterized all high-intensity infections. It was also noted that in higher-intensity infections the extensor surfaces yielded greater numbers of microfilariae than did the flexor surfaces.

TABLE 2

Subject Y-Y with high-intensity Onchocerca volvulus *infection, showing numbers of microfilariae recovered on skin-scarification smears from six sites in each of 16 different anatomical regions. Sites 1–3 were from the right side of the body, 4–6 from the left.*

Anatomical region	Sites						Total
	1	2	3	4	5	6	
Temple	0	0	0	1	0	0	1
Shoulder (ext.)	0	4	1	2	1	0	8
Chest (flex.)	0	0	0	2	0	0	2
Forearm (ext.)	24	24	6	0	3	0	57
Forearm (flex.)	1	8	2	0	1	2	14
Wrist (ext.)	13	15	1	0	6	5	40
Wrist (flex.)	9	11	8	0	0	0	28
Back (ext.)	0	4	2	1	7	1	15
Abdomen (flex.)	2	1	1	0	1	1	6
Hip	21	36	2	20	55	23	157
Thigh (ext.)	8	10	33	4	20	23	98
Thigh (flex.)	4	9	12	9	18	14	66
Calf (ext.)	30	20	78	28	89	16	261
Calf (flex.)	5	10	3	41	9	9	77
Ankle (ext.)	3	6	8	0	1	1	19
Ankle (flex.)	2	0	13	6	4	0	25

Table 3 gives the microfilariae recovery rates from an infection of relatively low intensity. Here it can be seen that 11 of the 16 anatomical regions showed microfilariae on at least one of the six smears and altogether 21 of the 96 sites were positive. The numbers of microfilariae per positive smear were small, ranging from one to three except in the ankle where seven were seen on each of two smears. In this subject, as was the case with the others with infections of low to moderate intensity, there was no indication that microfilariae were more numerous in the skin over the extensor than the flexor surfaces.

As in Experiment 1 it was found that the hips and lower extremities yielded the greatest numbers of smears positive for microfilariae.

EXPERIMENT 3

The purpose of this experiment was to investigate the day-to-day variations in numbers of microfilariae found in skin-scarification smears

TABLE 3

Subject McG with low-intensity O. volvulus *infection, showing numbers of microfilariae seen in scarification smears from six sites in each of 16 different anatomical regions. Sites 1–3 were from the left and 4–6 from the right side of the body*

Anatomical region	Sites						Total
	1	2	3	4	5	6	
Temple	0	0	0	0	0	0	0
Shoulder (ext.)	0	0	0	0	0	0	0
Chest (flex.)	0	0	0	1	0	0	1
Forearm (ext.)	0	0	0	0	0	0	0
Forearm (flex.)	0	0	0	0	0	0	0
Wrist (ext.)	0	0	1	0	0	0	1
Wrist (flex.)	2	0	0	0	0	0	2
Back (ext.)	1	0	0	0	0	1	2
Abdomen (flex.)	1	0	0	1	0	0	2
Hip	1	0	0	3	0	0	4
Thigh (ext.)	0	1	1	0	0	0	2
Thigh (flex.)	1	0	0	0	1	2	4
Calf (ext.)	0	0	0	0	0	0	0
Calf (flex.)	0	1	2	0	0	0	3
Ankle (ext.)	7	0	0	7	0	1	15
Ankle (flex.)	0	0	0	1	1	0	2

from closely placed sites in the same anatomical region. Smears were taken daily for 7 consecutive days from contiguous sites on both sides of the body at the same regions as described for Experiment 1. Ten subjects were studied in this manner.

Results. Numbers of microfilariae found in smears taken from closely placed sites on consecutive days often showed wide variations. In general, subjects with infections of higher intensities showed the widest variations in numbers of microfilariae recovered and, in one extreme example, smears from the ankle showed two microfilariae on the 2nd day, 265 on the 6th day and 11 on the 7th day. The findings in a representative subject with an infection of moderately high intensity are shown in Table 4.

By determining for each subject the total number of microfilariae found each day in the 14 smears it was observed that the total daily microfilaria-recovery rates varied greatly within the 7 days of the study. The subject in Table 4 showed total numbers ranging from 5 to 65 and in two other cases numbers ranged from 0 to 35 and 19 to 100, respectively.

TABLE 4

Subject C. *with* O. volvulus *infection showing numbers of microfilariae recovered on skin scarification smears made daily from contiguous sites for 7 consecutive days*

Day	Temple		Shoulder		Forearm		Wrist		Hip		Calf		Ankle		Total
	L	R	L	R	L	R	L	R	L	R	L	R	L	R	
1	0	0	2	2	1	3	0	1	12	6	0	0	27	11	65
2	0	0	1	1	0	0	0	0	2	0	0	1	2	0	7
3	0	0	0	0	0	0	0	0	3	1	0	0	1	3	8
4	0	0	0	0	0	0	0	0	3	14	1	1	26	7	52
5	0	0	0	0	0	0	0	0	3	6	5	11	4	15	44
6	0	0	1	2	0	0	1	0	14	2	1	3	20	5	49
7	0	0	0	1	0	0	0	1	0	0	0	1	1	1	5

TABLE 5

Subject L. *with* O. volvulus *infection showing unilateral concentration of microfilariae in ankle and calf as demonstrated by skin-scarification smears*

Day	Temple		Shoulder		Forearm		Wrist		Hip		Calf		Ankle		Total
	L	R	L	R	L	R	L	R	L	R	L	R	L	R	
1	0	0	0	0	0	0	0	0	1	3	2	0	8	0	14
2	0	0	0	0	0	0	0	0	7	1	0	0	4	0	12
3	0	0	0	0	0	0	0	0	5	1	0	0	5	0	11
4	0	0	0	0	0	0	0	0	2	2	3	0	8	0	15
5	0	0	0	1	0	0	0	0	7	1	3	0	4	0	16
6	0	0	0	0	0	0	0	0	7	2	5	0	0	0	14
7	0	0	0	0	0	0	0	0	2	4	4	0	8	0	18

Several subjects showed consistently higher microfilaria counts on smears from the lower extremities on one side than the other. Table 5 gives the results for one subject in whom smears from the right ankle and calf were consistently negative but showed a moderate infection-intensity for the left.

DISCUSSION

The results of the present studies on the skin-scarification technic show that the highest microfilarial recovery rates were found in the hips and lower extremities. An increased concentration of microfilariae in the skin of the lower half of the body was also reported by Kershaw,

Duke and Budden (1954) working with skin biopsies in onchocerciasis cases in Nigeria. On the other hand, Mazzotti (1951) found that in Mexico the highest numbers of positive skin biopsies occurred in the shoulder region. These reports indicate that the distribution of microfilariae in the skin of onchocerciasis cases in different regions of the world does not necessarily coincide.

In light infections it has been shown that only one of six smears made simultaneously from the same anatomical region may be positive and, in smears taken from contiguous sites on successive days, only one of seven daily smears may show microfilariae. The present study shows that, for diagnostic purposes, best results will be obtained in using the skin scarification technic by making more than one smear from each anatomical region, examining both sides of the body, and repeating examinations on successive days. Certainly, for this geographic area at least, it would be advisable to examine a minimum of four smears, one from each hip and calf.

The wide variations seen in numbers of microfilariae recovered from smears taken simultaneously from the same region, as well as on successive days from closely placed sites, may be explained by an inadequacy of the skin-scarification technic or it may indicate a true fluctuation in numbers of microfilariae occurring in space and time. Quantitative studies currently in progress by Dr. A. E. Gunders and the writer on microfilarial distribution patterns indicate that the wide variations in microfilarial recovery characteristic of the skin-scarification technic are due mainly to intrinsic limitations of the technic. Such factors as the length and depth of the incisions and the amount of pressure used to express the tissue juices cannot be accurately controlled. The skin scarification technic is not, therefore, considered suitable for precise quantitative studies, but can provide a rough measure of the microfilarial density and distribution.

In a previous study, the writer (Miller, 1955) found that, for the shoulder region, the skin-biopsy technic proved to be more efficient than the skin-scarification technic in the ratio of 3:2. In the series of cases studied by Mazzotti (1954) the skin biopsy proved to be only slightly more efficient, viz., 92 per cent of positive cases diagnosed as compared to 84 per cent by the scarification technic. Undoubtedly the microfilarial densities in the cases studied greatly influence the results obtained. In general, the efficiency of the skin-scarification technic increases with higher parasite densities and it follows that, in low-density infections, the efficiency of the skin-biopsy technic will be relatively greater.

SUMMARY

Skin-scarification smears were made from adult subjects infected with *Onchocerca volvulus* to compare the distributions of microfilariae in the skin at various anatomical regions and the variations in numbers occurring in multiple smears taken simultaneously, as well as on different days, from the same anatomical region.

The lower extremities and the hips showed the highest microfilarial-recovery rates and, for diagnostic purposes in Liberia, it is recommended that a minimum of four smears be made, one from each hip and calf.

Wide variations were seen in numbers of microfilariae recovered in smears taken simultaneously and on successive days from the same anatomical region. These variations are considered to be due mainly to limitations of the technic, which should not be used for quantitative studies. The skin-scarification technic, however, can provide a rough measure of microfilarial distribution and numbers in infected subjects.

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SOME CLINICAL ASPECTS OF ONCHOCERCIASIS IN LIBERIA

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The studies of Burch, Qualls and Greenville (1955) demonstrated that infections with *Onchocerca volvulus* are widespread in Liberia and that ocular pathology attributable to these infections does not constitute a serious health problem.

In the present investigation adult subjects were studied in an attempt to relate the presence of infection to clinical signs and symptoms, particularly to skin changes.

MATERIALS AND METHODS

A total of 125 adults 16 years of age or older, were selected at random for this study. The group included 81 males and 44 females. They were employees, or dependants of employees, of the Firestone Plantations Company and lived in an area where onchocerciasis was endemic. The time spent on the plantation by the different individuals ranged from a few days to many years and there was no way of determining the duration of infections, many of which undoubtedly were acquired elsewhere.

Subjects were brought to the clinic for examination. They were stripped of all clothing and examined for skin changes and the presence of nodules. Slit-lamp and ophthalmoscopic examinations were made to determine the occurrence of eye pathology and the presence of free-swimming microfilariae in the eye chambers. In addition, the frequence of hydroceles in males was investigated and a history of births, miscarriages and abortions was obtained from the women.

The presence of infection was determined by examination of skin-scarification smears and by palpation for nodules. Fourteen skin scarification smears were taken from each subject, one on each side of the body at the following seven levels: (1) temple, near outer canthus; (2) shoulder, over scapula; (3) mid-forearm, outer lateral surface; (4) wrist, extensor surface; (5) greater trochanter; (6) mid-calf, outer lateral surface; (7) ankle, outer lateral surface.

The skin-scarification technic involved making five superficial, linear incisions about 1 mm apart and 1 to 2 mm long. Blood and tissue juices were expressed from the incisions by manual pressure, collected on a slide and stained with Giemsa stain or hematoxylin. Smears were searched microscopically for microfilariae with the 16-mm objective and diagnosis was confirmed with the oil-immersion lens.

RESULTS

Infection rates. The results of scarification-smear examinations and nodule palpations are shown in Table 1. A study of the table shows that 67 (53%) of the subjects were positive for microfilariae and that there were also 19 in whom microfilariae were not found but in whom the presence of nodules characteristic of onchocercomata was demonstrated. The total infection rate was thus established at 69 per cent. The infection rates in males and females were almost identical, being 69 per cent and 68 per cent, respectively.

Although it was not possible to determine the ages of the subjects, they were divided into two groups on the basis of appearance: 16 to 39 years, and 40 years and over, respectively. Infection rates in these two groups are shown in Table 2 where it is seen that all subjects estimated as 40 years or older were found positive for infection. The younger age group showed an infection rate of 59 per cent.

Skin changes. Nine of the 125 subjects examined showed the well-defined skin changes considered to be characteristic of onchocerciasis. Seven of these were found positive for infection by demonstrating microfilariae in skin-scarification smears while, in the remaining two, typical nodules were found. It was possible to re-examine one of these two cases at a later date and microfilariae were seen in a skin biopsy from the hip. Skin changes were seen more frequently in the older age group. Five (16%) among 31 of these showed skin changes as compared to four of 55 (7%) among the younger group. In none of the 39 individuals negative for either microfilariae or nodules was characteristic skin change observed.

Other clinical findings. Ocular lesions attribut-

TABLE 1

Infection rates of O. volvulus *in Liberian adults*

Sex	No.	Micro-filariae pos.	Nodules only	Total pos.	Percent pos.
Males	81	44	12	56	69
Females	44	23	7	30	68
Total	125	67	19	86	69

TABLE 2

Infection rates of O. volvulus *in Liberian adults 16 to 39 years of age as compared with those in persons 40 years or older. Figures in parenthesis show percentages*

Age	Males		Females		Totals	
	Exam.	Pos.	Exam.	Pos.	Exam.	Pos.
(*years*)						
16–39	57	32 (56)	37	23 (62)	94	55 (59)
40+	24	24 (100)	7	7 (100)	31	31 (100)

able to onchocerciasis were not seen in any member of the infected group, nor were free-swimming microfilariae observed in the chambers of the eye.

Five of the 81 males had hydroceles, four unilateral and one bilateral. Three of the 56 males with onchocerciasis were found with hydroceles while the uninfected group of 25 showed two with hydroceles, one of which was bilateral.

Studies of pregnancy histories showed that 30 women with onchocerciasis gave a history of 98 pregnancies. Of these, 31 or approximately 30 per cent, terminated in abortion or miscarriage. The comparative figures for the 14 uninfected subjects were 38 pregnancies with 15, or approximately 40 per cent, terminating in abortion or miscarriage.

DISCUSSION

While there can be no doubt about a positive diagnosis when the characteristic microfilariae of *O. volvulus* are found, there may be some question about basing a diagnosis on the presence of nodules only. Burch *et al.*, (1955) in a restudy of 66 patients in whom nodules were palpated but in whom microfilariae were not seen, were able to demonstrate in all cases the presence of microfilariae in excised nodules or nodule aspirates. In our studies it was possible to re-examine, by skin biopsy of the hip, nine of the 19 subjects with nodules but negative for microfilariae on scarification smears. All nine were found positive for microfilariae. The remaining ten cases with nodules would probably have shown microfilariae had repeated examinations been possible.

Infection rates found in the present study are considerably higher than those reported by Burch *et al.*, (1955) in their survey. This may be explained in part by age differences in the groups examined. A further factor may be diagnostic technics employed. The latter workers examined skin-scarification smears and biopsies taken from the shoulders only and it has been shown (Miller, 1955, 1956) that in the area studied a number of positive cases will be missed if examination for microfilariae of the skin from the pelvic region and lower extremities is omitted. It is of interest to note that in the present study there were 18 subjects positive for microfilariae in whom nodules could not be demonstrated.

The problem of ascribing skin changes to *O. volvulus* infections in this region is not an easy one because skin abnormalities due to nutritional deficiencies are of common occurrence. A frequent finding is atrophy of the skin over the shin which has a smooth, burnished look. Another change commonly found is a roughened, dry and thickened appearance of the skin over the knee and the adjoining regions of the thigh and leg. These changes, seen in both infected and non-infected cases are considered to be due to a combination of factors including faulty nutrition, trauma, previous skin diseases and possibly exposure. On the other hand, nine subjects showed well-defined skin changes characterized by a dry, atrophic, sometimes roughened appearance, with mosaic fissuring which in advanced cases could be classified as 'lizard skin'. The changes were best seen on the lower extremities, buttocks, lower back and extensor surfaces of the arms. As all nine were found to be infected with *O. volvulus*, it was considered justifiable to relate these changes to the presence of the parasite.

The reports of Strangway and Strangway (1951), Ikejiani (1954) and others indicate that onchocerciasis may be a factor in habitual abortion. The occurrence of *O. volvulus* microfilariae in hydrocele fluid raises the question of a possible relationship between hydrocele formation and this infection. According to Dyce Sharp (1927) onchocerciasis can cause a characteristic hydro-

cele which differs from that due to *Wuchereria bancrofti*. Kirk (1947) reported a high incidence of hydrocele and elphantiasis in the Sudan where infections with *O. volvulus* are common but where *W. bancrofti* is extremely rare. While the series of cases in the present investigation is small our data indicate that hydroceles in our area are seen as frequently in uninfected males as in those with onchocerciasis, and that abortion rates are at least as high in uninfected as in infected females.

SUMMARY

Clinical and laboratory studies were carried out on 125 adults (81 males and 44 females) chosen at random and living in an area in which onchocerciasis was endemic. Special attention was given to skin changes, signs of eye pathology and the occurrence of miscarriage or abortion in the females and hydrocele in the males. Positive diagnoses were made by examination of 14 scarification smears from each subject and by palpation for onchocercomata.

Overall infection rate for *O. volvulus* was 69 per cent and in the group 40 years and older it was 100 per cent. Specific skin changes were found in 14 per cent of the infected subjects. No ocular pathology attributable to onchocerciasis was seen. There was no evidence to suggest that in Liberia onchocerciasis was responsible for an increased rate of miscarriages or abortions or that it was a factor in hydrocele production in the male.

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TECHNIQUES FOR HEMAGGLUTINATION AND HEMAGGLUTINATION-INHIBITION WITH ARTHROPOD-BORNE VIRUSES

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During the past few years, the methods for conducting hemagglutination (HA) and hemagglutination-inhibition (HI) tests with arthropod-borne (arbor) viruses have undergone a number of modifications, directed toward simplification and improvement as well as extension of the tests to include a larger number of arbor viruses. Accordingly, it seems desirable at this time to bring together, in one publication rather than in a series of papers, a description of the procedures currently in use in The Rockefeller Foundation Virus Laboratories. Some of the procedures were initially developed elsewhere; of those developed in this laboratory, most aspects have not been previously reported.

It was stated by Hallauer (1946) that he had obtained a hemagglutinating antigen from viscerotropic but not from neurotropic strains of yellow fever. It was, however, Sabin and his associates (Sabin and Buescher, 1950; Sabin, 1951; Chanock and Sabin, 1953, 1954a, 1954b; Sweet and Sabin, 1954) who first demonstrated HA with a number of arbor viruses. Their results with Japanese B, St. Louis, West Nile, Russian spring-summer, Western equine encephalomyelitis and dengue viruses indicated that this might be a general property of the arbor group. In addition, these investigators showed that arbor virus hemagglutinins had rather specific requirements as to pH and type of erythrocyte. Casals and Brown (1954), by the use of acetone- and ether-extracted antigens, greatly extended the list of arbor viruses showing HA activity and found, through the HI test, that most of the known arbor viruses fall into immunologically distinct groups of which three are now recognized, A, B and C (Casals, 1957). Further contributions to the methodology were reported by Porterfield (1954, 1957), Clarke and Casals (1955), and Clarke and Theiler (1955).

It is not the purpose of this communication to discuss in detail the applications of the HA-HI techniques. Their use in the systematic classification of arbor viruses has been mentioned above, and from this a closer understanding of the relationships between the various viruses and diseases is beginning to emerge. The HI test is of considerable help in virus identification and in serologic diagnosis of infection, although the immunologic relationships between viruses of the same group may complicate the interpretation of such tests. It is becoming increasingly evident, however, that this problem is common to all types of serologic procedures and that differences in specificity between results obtained by HI, complement-fixation and neutralization tests are relative rather than absolute. Immunologic overlap must, therefore, be kept in mind in the interpretation of serologic data obtained by any of the commonly used procedures.

PREPARATION OF ANTIGENS

All the viruses at present recognized in Groups A, B and C have the capacity to give rise to HA antigens; some viruses outside these groups also have this property. It should be stressed here, however, that there is still a substantial number of arbor viruses which have not yielded an HA antigen by the current methods. Furthermore, the possibility should not be overlooked that some strains of a given virus may show greater capacity than others to yield an HA antigen.

In Table 1 are listed a majority of the arbor viruses for which HA antigens have been prepared. The list is incomplete in that a number of recently isolated hemagglutinating viruses have been omitted as well as a few others, such as louping ill, which have not yet been examined for their HA activity. The viruses are listed according to their serologic group, if any, and the best source and methods for preparing each antigen

TABLE 1

Preparation of arbor virus hemagglutinating antigens

Group	Virus	Source*	Methods§	
			Preferred	Alternative
A	Chikungunya	smb	1P	
	Eastern equine encephalomyelitis	smb	1	
	Mayaro	sms	1	
	Mayaro	smb	1P	
	Middelburg	smb	1P	1
	Semliki Forest	smb	1P or 1	
	Semliki Forest	sms	1	
	Sindbis	smb	1	
	Western equine encephalomyelitis	smb	1	
	Venezuelan equine encephalomyelitis	smb	1	
B	Bat salivary gland	smb	1	Not studied
	Dengue type 1 (Hawaiian)	smb	1 or 1P	
	Dengue type 2 (New Guinea B)	smb	2P or 2	1
	Dengue type 2 (Tr 1751)	smb	1	
	Ilheus	smb	1	3
	Japanese B	smb	1	3
	Kyasanur Forest Disease	smb	1P	1, 3P
	Murray Valley	smb	1	3P
	Ntaya	smb	1	Not studied
	Omsk Hemorrhagic Fever	smb	1P	
	Russian spring-summer	smb	1P	1, 3P
	St. Louis	smb	1	3 or 3P
	Spondweni	smb	1	Not studied
	Wesselsbron	smb	1	Not studied
	West Nile	smb	1	3
	Uganda S	smb	1 or 1P	Not studied
	Yellow Fever-Asibi strain	Rms†	1	
	Yellow Fever-Asibi strain	smb	1	
	Yellow Fever-French viscerotropic	smb	1	Not studied
	Yellow Fever-French neurotropic	smb	1	Not studied
	Yellow Fever-JSS	smb	1	Not studied
	Yellow Fever-17D	smb	2P	3P
	Zika	smb	1	Not studied
C	Marituba	sms	1	
	Marituba	sml	2	
	Oriboca	sms	1	
	Oriboca	sml	2	
	Caraparu	sms	1	Not studied
	Murutucu	sms	1	Not studied
	Apeu	sms	1	Not studied

TABLE 1—*Continued*

Group	Virus	Source*	Methods§	
			Preferred	Alternative
None	Bunyamwera	smb	2	
	Bunyamwera	sms	1 (inconsistent results)	
	California encephalitis	smb	2	
	Tr 3587	smb	2	
	Phlebotomus Fever (Naples strain)	smb	2	
	Phlebotomus Fever (Sicilian strain)	smb	2	
	Rift Valley Fever	ams‡	Storage at −24°C	1

* smb = suckling-mouse brain; sms = suckling-mouse serum; Rms = rhesus monkey serum; sml = suckling-mouse liver; ams = adult-mouse serum.

† First described by Porterfield, 1954.

‡ Mims and Mason, 1956. This virus not studied at RFVL.

§ 1 = extraction with acetone and ether
2 = sucrose-acetone extraction
3 = alkaline aqueous suspension
P = protamine treatment of final antigen

are given. Where more than one source or method of preparation have been shown to yield good antigens, this fact is indicated. The methods are designated by numbers in the table, the key being given at the end of the table. A full description of each method follows in the text. In certain instances, a choice is given as to the use of protamine sulfate. This is because some antigens give satisfactory results in lucite plates without protamine treatment even though their performance in glass tubes is poor unless they have been so treated.

SOURCE

The most generally useful source of HA antigens for arbor viruses is the brain of the suckling mouse. Most of the viruses attain a significantly higher titer in the very young than in the adult mouse and, in some instances, this difference is very great. In addition, it seems probable that the chemical changes associated with growth and development make it more difficult to demonstrate hemagglutinating activity in the brains of older animals.

Mice are inoculated by the intracerebral route for the production of brain antigens, the dose usually being 0.02 ml of a 10^{-2} dilution of stock infectious suckling-mouse brain. The age of the mice used is varied according to the incubation period of the virus; 4- to 6-day-old mice are used for viruses with short incubation periods (1–2 days), 3- to 4-day-old mice for medium-incubation-period viruses (3–4 days), and 1- to 2-day-old mice for viruses with longer incubation periods. Conditions are adjusted so that the brains can be harvested at a time when some of the mice are beginning to die and the rest appear sick from the infection. The animals are anesthetized with ether and partially exsanguinated by cutting through the chest wall with small scissors, after which the brains are removed and transferred to a small, weighed, glass preparation dish (5 x 3 cm) with fitted glass cover held in a bath of CO_2 ice and alcohol. An alternative and more rapid method is to aspirate the brain directly from the unopened skull by means of a large (13–15 gauge) needle attached to a vacuum system as described by Strome (1953). During the aspiration step, the animal is held in the hand of the operator; consequently, although such a closed system should decrease the exposure of personnel to infectious brain material, when it is used following partial exsanguination of the animal by cutting open the chest cavity it may be associated with increased hazard of exposure to potentially or demonstrably infectious blood. After determination of the brain weight, the container may be sealed with Scotch and adhesive tape and stored in a CO_2-ice cabinet until used.

As is apparent from Table 1, an alternative source for some HA antigens is serum. Although it may yield antigens of better quality than those obtained from brain, serum is of limited usefulness owing to the fact that relatively few arbor viruses reach a sufficiently high titer in the blood of common laboratory animals. The Asibi strain of yellow fever virus reaches an adequate titer in the serum of the rhesus monkey, and Rift Valley fever virus in the serum of the adult mouse. Certain other virus antigens can be prepared from the serum of the suckling mouse, but this source is obviously inconvenient from the standpoint of yield. The development of alternative methods for antigen preparation and improvements in the test procedures have increased the number of viruses yielding satisfactory antigens from suckling-mouse brain. The C group viruses, however, show a higher infective titer in extraneural tissues, such as blood and liver, than in brain, which fails even as a satisfactory source for complement-fixing antigens. In this group, suckling-mouse serum is the best HA antigen source discovered so far. Nevertheless, hemagglutinating antigens have been prepared from suckling-mouse liver by the sucrose-acetone procedure for two members of the C group (Marituba and Oriboca); other viruses have not been studied. Acetone-ether extraction of suckling-mouse liver provides good CF antigens, but these are devoid of detectable HA activity.

The route of inoculation of suckling mice for the production of serum antigens does not appear to be critical, similar results having been obtained following intraperitoneal and intracerebral inoculation. The dose should be so adjusted that the animal does not succumb to infection before a high level of viremia is obtained. Mice are used at as old an age as possible to obtain a larger blood yield, but since the level of circulating virus tends to decrease sharply with increasing age, there is relatively little latitude and mice must usually be infected within the first week of life. The optimal time for bleeding (time of peak viremia) depends upon the virus employed; for C group viruses, this varies between 24 and 48 hours after inoculation. Although blood is easily collected from adult mice by heart puncture through the closed chest of the anesthetized animal, this procedure is not suitable for such small animals as suckling mice. The most rapid procedure is to cut through the anterior chest wall and the heart of the anesthetized animal with small, sharp-pointed scissors and allow the blood to drop into a small tube held in an ice-water bath. A better yield can be obtained by aspirating the blood gently into a 0.25-ml syringe without a needle as it collects in either (1) the open chest cavity after the heart is nicked with sharp scissors, or (2) a posterior axillary skin flap after the axillary vessels are severed. The blood is transferred to a tube held in an ice bath. Serum is separated from the clot and may be processed at once or after storage in sealed ampules in a CO_2-ice cabinet.

METHODS

(*1*) *Extraction with acetone and ether.* The acetone and ether are analytical-reagent-grade chemicals. In a few trials, anesthesia-grade ether was also found satisfactory. These reagents are chilled in an electrical deep freeze ($-15°C$) prior to use. All work is carried out in ice baths, and centrifugation is done in the cold room or in a refrigerated centrifuge adjusted to 0°C.

For preparation of brain antigens, homogenization may be carried out in a conventional Waring blendor, but the recommended procedure is to use a homogenizer equipped with an overhead drive and a side port at the bottom through which the homogenate may be removed without opening the vessel. Stainless steel homogenizers of the overhead drive type can be obtained from the Lourdes Instrument Corporation[1]. The safety side port, which can be added by a skilled metal worker, consists of a small opening at the base of the vessel which is closed by a gum-rubber diaphragm held in place by a hollow, threaded, metal cylinder. The cylinder, in turn, is closed by a cap. The contents of the homogenizer can be rapidly aspirated into a 200-ml centrifuge bottle using a closed system. The centrifuge bottle is closed with a two-hole rubber stopper fitted with bent glass tubing through each hole. A 16- or 17-gauge needle is attached by gum-rubber tubing to one glass tube, and gentle suction from a water pump is applied to the other tube. The rubber diaphragm of the side port of the homogenizer is pierced by the needle and, *after suction is established*, the homogenizer top is slightly

[1] Lourdes Multi-mixer. Lourdes Instrument Corporation, 53rd Street and First Avenue, Brooklyn 32, New York.

loosened to permit entrance of air. The homogenizer is never opened until after it has been autoclaved or otherwise decontaminated. If homogenization is carried out in a blendor without a side port, the blendor contents are poured from the open vessel into centrifuge bottles, care being taken to assure equal distribution of the solids in all the bottles.

The weighed, solidly frozen brain is thawed just sufficiently to loosen it from its container and is then dropped into the chilled homogenizer cup. Chilled acetone is added in an amount which is 20 volumes per unit weight of brain. The homogenizer is closed and, if it is an overhead-drive type, is immersed in an ice bath. Homogenization is carried out once or twice for 1-minute intervals, the homogenate is transferred to one or more 200-ml centrifuge bottles which are then closed with gum-rubber stoppers, and centrifugation is carried out for 5 minutes at 1,800 rpm in the cold. The supernatant fluid is aspirated off and fresh acetone added to the sediment in an amount equal to the original volume. The sediment is suspended by vigorous shaking, and the bottle is held for 20 minutes in an ice bath with occasional shaking. Centrifugation is then repeated as before, and extraction is carried out three more times, each for 20 minutes, once with a mixture of equal parts acetone and ether and twice with ether alone. If the material is distributed in more than one bottle, the sediments from all bottles are pooled at the end by washing over with small volumes of ether and recentrifuging. After almost all of the final supernatant ether has been removed by aspiration, the bottle is closed with a rubber stopper fitted with glass tubing and connected to a water pump. We find it helpful to add a few glass beads prior to drying. The residual ether is then evaporated off while the tube is swirled to facilitate drying and avoid bumping. The bottle is then connected to a Hyvac oil pump and drying is continued for 1 hour with the bottle immersed in an ice bath. The pump is protected from contamination by a trap containing a large quantity of nonabsorbent cotton. This trap is autoclaved after each use.

In order to dispose of the supernatant fluid, after each centrifugation it is aspirated into a large suction flask connected to a water pump. During the subsequent extraction period, the volatile solvents are evaporated via the water pump by clamping the open rubber tubing and placing the flask in a pan of hot water. At the end of the procedure, the dry residue in the flask is decontaminated by autoclaving.

The dried, extracted brain is rehydrated by the addition of 2 ml of chilled 0.9 per cent NaCl per 1 gram of original brain. The saline may be added directly through the glass tubing by means of a long needle attached to a syringe. If wet gauze is held around the needle, the hazard of dispersing the dry material into the open air is diminished. After the powder has been wetted to some degree, a solid stopper is substituted and the bottle is shaken vigorously at intervals to facilitate rehydration. The bottle is held overnight in an ice-water bath in a cold room. On the following day, the suspension, which should be well dispersed, is transferred to lusteroid tubes and centrifuged for 1 hour at 10,000 rpm (11,000 G). The supernatant fluid constitutes the antigen and is distributed in 0.5-ml to 2.0-ml volumes in ampules, lyophilized and stored in an electrical deep freeze (−15°C). Such preparations show great stability over periods of months or years.

Acetone-ether extraction of serum is carried out in much the same way as for brain with a few exceptions. The serum is added dropwise from a syringe through a small-gauge needle into 20 volumes of chilled acetone, the mixture being kept in constant motion during the addition either by swirling the bottle or by stirring with a motor-driven rod. Centrifugation, re-extraction and drying are the same as for brain antigens. The dried extracted serum is rehydrated in 5 volumes of borate-saline, pH 9.0 (see section on chemical reagents). The extracted serum goes readily into solution, it is unnecessary to leave the preparation overnight, and centrifugation of the finished antigen is not required. As with brain preparations, serum antigens are preserved by lyophilization and storage at −15°C.

It might be mentioned that, in the absence of facilities for lyophilization, finished antigens are best stored in the wet frozen state in a CO_2-ice cabinet. Brain antigens, at least, may deteriorate within a few weeks at −15°C.

(*2*) *Sucrose-acetone extraction.* This procedure is proving superior to all of those tried so far in that by its use antigens can be prepared with certain viruses or from some tissues when other methods fail. Furthermore, even in situations where other methods are effective, the sucrose-

acetone technique often gives preparations that are markedly better. The reason for not using it exclusively is that the handling of the required large volumes of acetone makes the procedure somewhat inconvenient when more than small yields of antigen are desired. It is of interest that this method may also yield complement-fixing antigens of significantly higher titer than those obtained by acetone-ether extraction. The role of sucrose appears to be a protective one against the action of acetone. It has been demonstrated, with Bunyamwera virus, that sucrose also protects the infective property significantly, though not completely, against the inactivating effect of acetone, so that such antigens may be much more highly infectious than those prepared by acetone-ether extraction. Such preparations should, therefore, be handled with due care. A number of attempts have been made to modify the procedure to make it more convenient, but these have met with no success. The relationships between the sucrose, brain, water and acetone concentrations appear to be rather critical.

The infected suckling-mouse brain is homogenized with 4 volumes of a chilled 8.5 per cent aqueous solution of sucrose. The whole homogenate is added dropwise, with brisk mechanical stirring, to 20 volumes of chilled acetone. The acetone must be in a proportion of 20 volumes to 1 volume of *homogenate*. The mixture is shaken vigorously and centrifuged for 5 minutes at 1,800 rpm. The milky supernatant fluid is aspirated off, the sediment at this stage being a pink, gummy mass which is tightly adherent to the bottom of the centrifuge bottles. A volume of fresh acetone equal to that originally used is added to each bottle, and the preparations are allowed to stand in ice baths for at least 1 hour to dehydrate the sediment. After sufficient time, the sediment is readily reduced to a fine suspension by use of a thick glass rod or, preferably, a motor-driven plunger. The bottles are then centrifuged as before, the supernatant fluid is discarded and the sediments from all bottles are pooled in one with fresh acetone. Centrifugation is carried out as before and, after removal of the supernatant acetone, the sediment is dried as in method 1. To the dry powder, a volume of saline is added which is equal to 0.4 of the total volume of homogenate used. Owing to the presence of the sucrose, this preparation rehydrates more readily than does the acetone-ether extracted powder, and the antigen may be completed after standing for about 1 hour; if not, it can be left overnight in ice as in method 1. It is then centrifuged for 0.5 to 1 hour at 10,000 rpm. The supernatant fluid is the antigen. It is lyophilized and stored as in method 1.

(*3*) *Alkaline aqueous suspension.* Many, if not all, Group B viruses show some degree of hemagglutinating activity in crude 10 to 20 per cent alkaline suspensions after moderate centrifugation. In some cases, the titers and pH range of activity can be considerably improved by protamine treatment of the supernatant fluid. Homogenization is carried out in borate-saline, pH 9.3, and centrifugation is for 1 hour at 10,000 rpm; if protamine is to be used, centrifugation may be done at 2,500 rpm. The preparations are stable for considerable periods of time in sealed ampules in the CO_2-ice cabinet. Their stability to lyophilization has not been determined. These suspensions are of particular value in the rapid characterization of Group B virus isolates or in special studies where it is desirable to have high infective and HA titers in the same preparation. Acetone or acetone-ether extraction has been the preferred technique for antigen production in these laboratories because of its wide applicability to arbor viruses of all groups. Alkaline aqueous suspensions have not been used, by us, as routine HI antigens, and we are unable to make any statements concerning their reactivity as compared with extracted antigens.

Protamine sulfate treatment. Brain-tissue antigens prepared from some viruses by any of the methods described may prove unsatisfactory in practice. For example, the pH range over which hemagglutination occurs may be excessively narrow. Or, the HA patterns may be so poor as to interfere with the interpretation of HI tests. These difficulties tend to be more marked in tests carried out in glass tubes than in those done in lucite plates. Treatment of the finished antigen with protamine may serve to broaden the pH range, particularly toward the acid side, and to improve the pattern. Depending on the virus and preparation, the improvement may be associated with no change, an increase, or a decrease in the HA titer at the optimal pH. In a few cases, the decrease in HA titer has been rather marked and, accordingly, where protamine is to be tried with new viruses, the preparation should be tested for HA activity both before and after protamine

treatment. Warren *et al.*, (1949) previously described the use of protamine sulfate in the partial purification of the infective property of certain arbor viruses.

Protamine sulfate[2] is prepared in 0.9 per cent NaCl at a concentration of 50 mg/ml of NaCl for use with extracted antigens and 20 per cent alkaline aqueous suspensions or 25 mg/ml for 10 per cent alkaline aqueous preparations. It is prepared shortly before use and is turbid rather than clear. The antigen is chilled in an ice bath and treated by the addition of 0.1 volume of the appropriate protamine preparation. A dense white precipitate begins to form at once. The mixture is allowed to stand in the ice bath for 30 minutes, with occasional shaking, and is then centrifuged in the cold for 15 minutes at 2,500 rpm. The clear supernatant fluid is the treated antigen. Protamine presumably precipitates inhibitory substances and those which tend to distort the HA pattern. Protamine-treated extracted antigens are lyophilized and stored at −15°C; treated aqueous homogenates are stored in a CO_2-ice box.

A word of caution should be added in connection with the use of protamine sulfate for HA antigen preparation. Protamine itself has hemagglutinating activity which is most manifest at low pH (6.0–6.4) and low temperature (4°C). This protamine effect appears to be inhibited rather readily in the presence of low concentrations of added bovine plasma albumin. Although an excess of protamine is necessary for effective antigen treatment, from a practical standpoint this creates no difficulties if care is taken to maintain some degree of balance between the concentrations of tissue components and of protamine. The residual protamine in the supernatant fluid can, of course, be removed with heparin, but this additional step makes the procedure quite expensive and, in our limited experience, may result in some loss in HA titer. In any case, HA activity that manifests itself only after protamine treatment and at low pH must be viewed with suspicion until demonstrated to be viral in nature.

PREPARATION OF SERA

Standard immune sera. It is advantageous to have on hand high-titer reference immune sera prepared against all viruses under study. Although such sera can be prepared in any laboratory animal demonstrated to give a satisfactory immune response, the adult mouse has been used most generally because the same serum then serves for both HI and complement-fixation work without the introduction into the latter test of the complication of antibodies against mouse brain. Mice are immunized by repeated intraperitoneal inoculations of infected suckling-mouse-brain suspension prepared in saline or phosphate buffered saline. For those viruses which produce lethal infections in mice by peripheral routes, the first two inoculations are of formalin-inactivated virus, followed after 1 week by one to three inoculations of untreated infectious virus. Each dose consists of 0.1 ml of 10 per cent mouse brain. The mice are bled about 1 week after the last inoculation.

Preparation of sera for tests. As was first observed by Sabin and his associates, all sera appear to contain nonspecific inhibitors of arbor virus hemagglutinins. These are often present to very high titer and their removal is, of course, a prerequisite to antibody study by the HI procedure. In addition, sera frequently contain naturally occurring agglutinins for the type of erythrocyte (goose) now used in the test and such agglutinins must also be removed.

The available evidence suggests that the nonspecific inhibitors are lipid or lipoprotein in nature. Two methods for their removal are recommended: adsorption with kaolin, designated as the routine, and extraction with acetone, designated as the reference procedure. The two methods have yielded essentially identical results with normal and immune sera from man, rhesus monkeys, Swiss mice, guinea pigs, rabbits and chickens. Treatment with kaolin is entirely empirical and is based on selective adsorption under standardized conditions. It has the great advantages of simplicity and speed. Extraction with acetone is somewhat tedious to carry out and is not well suited to the handling of large numbers of specimens. It is, however, an all-or-none procedure based on the solubility of the lipid inhibitors and the insolubility of the protein antibodies in acetone. There seems no reason to believe that variations in the chemical constitution of sera from different species alter this type of separation. Evidence that such variations can influence a procedure based on selective ad-

[2] Obtained from Nutritional Biochemicals Corp., Cleveland 28, Ohio.

sorption was obtained when bentonite was used as the adsorbent instead of kaolin; either nonspecific inhibitor was not entirely removed, or there was serious loss of antibody following treatment of sera of some species under conditions that were found satisfactory for other species. For this reason, it is strongly recommended that a comparison of the results obtained with the acetone and kaolin methods be made with normal and immune sera from species other than those listed above, or in any case where HI results after kaolin treatment appear open to question.

Routine kaolin method. A "25 per cent" suspension of acid-washed kaolin[3] is prepared by adding, with constant mechanical stirring, 25 g of the powder to 100 ml of borate-saline, pH 9.0. So far as is known, this slurry keeps indefinitely at 4°C. To 1 volume of undiluted test serum are added 4 volumes of borate-saline and 5 volumes of 25 per cent kaolin. The mixture is held at room temperature for 20 minutes with occasional shaking and is then centrifuged at 2,500 rpm, also at room temperature, for 30 minutes. The supernatant fluid is at pH 9 and is considered to represent a 1:10 dilution; the inaccuracy introduced by not taking into account the volume occupied by the solid kaolin is disregarded. If, because of inadequate quantity, it is necessary to treat sera that are at higher initial dilutions than 1:5, this may be done by using correspondingly more dilute suspensions of kaolin.

Reference acetone extraction method. For acetone extraction, the serum is diluted 10-fold with isotonic NaCl. We have used as little as 0.15 ml of whole serum without difficulty. The diluted serum is chilled in an ice bath, and 12 volumes (referred to the volume of diluted serum) of cold acetone are added. Extraction is carried out for 5 minutes in the ice bath, with periodic shaking, and the tube is centrifuged for 5 minutes in the cold at 2,000 to 2,500 rpm. After aspirating off most of the supernatant acetone, the precipitate is re-extracted with the same volume of acetone, vigorous shaking, with the tube stoppered, being necessary to resuspend the pellet. After recentrifugation and removal of the bulk of the acetone, the extracted serum is dried at room temperature with a water pump. During the initial phase, which is done individually for each sample, the tube is agitated to avoid bumping and loss of material. After all visible acetone has been removed, several tubes can be connected to the pump in series by the use of T tubes, and drying is then continued for about 15 minutes for small samples or for a longer period if the volume of serum used was large. The dried material is dissolved in a sufficient volume of borate saline, pH 9.0, to give the equivalent of a 1:10 serum dilution. Solution is easily effected by leaving the preparation overnight in the icebox or for an hour or two at room temperature with occasional agitation of the tubes.

Where the amount of serum available is less than 1.5 ml of a 1:10 dilution, acetone extraction can still be used if bovine plasma albumin is added as a "carrier" to provide a sufficient bulk of precipitate to assure quantitative recovery of antibody. Presumably, a small amount of normal serum could be substituted for bovine albumin if desired.

Removal of agglutinins. Since many sera contain naturally occurring agglutinins for goose erythrocytes, all sera are routinely adsorbed with such cells prior to use in tests. There is some evidence, as yet incomplete, to indicate that acetone-extracted sera may not require this step. The kaolin-treated sera are chilled in an ice bath to avoid the action of hemolysins, which also may be present in the sera. To each serum is added 0.1 ml of packed washed goose erythrocytes per 5 ml of serum at the 1:10 dilution. Adsorption takes place for 20 minutes with occasional shaking, after which the tubes are centrifuged for 10 minutes at 1,500 rpm in the cold. The supernatant fluids are now ready for use in the HI test.

PREPARATION OF ERYTHROCYTES

Until recently, the very young (approximately 1-day-old) chick was the only known source of satisfactory test erythrocytes. Porterfield (1957) reported that cells from the adult, white, domestic goose (*Anser cinereus*) were superior to chick cells and obviously considerably more convenient to obtain. Goose cells are now routinely used in this laboratory, and no variation in cell reactivity from individual bird to bird has been observed.

Under certain conditions of storage, erythrocytes show a tendency to shrink, hemolyze and lose reactivity. The method currently used, which appears to minimize or eliminate these difficulties,

[3] Obtained from Amend and Co., New York, N. Y. Specify acid-washed product.

is patterned after that described by Stein (1952) for the preservation of sheep erythrocytes.

In the following account, the cells are obtained and handled under sterile conditions. The bird is bled, from the wing or jugular vein or from the heart, into a syringe containing 1.5 ml of ACD (acid-citrate-dextrose—see section on chemical reagents) per 8.5 ml of blood. The cells are washed four times with DGV (dextrose-gelatin-veronal—see section on chemical reagents), the first time by adding 1 volume of whole blood to 2.5 volumes of DGV and subsequently with 3 volumes of DGV per original volume of whole blood. Centrifugation is for 15 minutes at 1,500 rpm in the cold. After the final washing, the cells are again suspended in 3 volumes of DGV and standardized. It is desirable to have a constant number of cells in each test since HA titer is dependent upon the number of cells present. Standardization is most easily achieved by determination of the optical density (O.D.), which is directly proportional to the cell count. The cells are standardized and stored at a predetermined concentration which is much (24–40 fold) higher than that used in the test. This provides a stock suspension of cells which can be diluted easily to give any desired optical density or pH without a perceptible alteration in the test conditions. The method used by us is to remove a small aliquot of the DGV cell suspension and dilute it 1:40 in 0.9 per cent NaCl. The O.D. of the dilute suspension is determined in a Coleman Junior Photoelectric Spectrophotometer at wave length 490 mμ and in tubes which are 10 mm in internal diameter. It is important to keep in mind that the reading obtained is proportional to the length of the light path through the cell suspension and will therefore be greater if tubes of larger diameter are used. The instrument is adjusted to 100 per cent transmission, with a similar tube containing saline as the blank. When both the total volume of the DGV suspension and the above optical density are known, it can be determined how to dilute the cells to give any desired concentration (final volume = initial volume X observed O.D./desired O.D.).

We adjust the suspension so that a 1:40 dilution gives an O.D. of approximately 0.450. Such a suspension is approximately 8 per cent by volume for goose cells and 10 per cent by volume for chick cells. Over a period of at least 3 weeks' storage at 2 to 4°C, DGV cell suspensions maintain a constant optical density (do not change in size), show minimal or no hemolysis and exhibit HA reactivity and patterns which compare very favorably with those of fresh cells. The standardized suspension is now ready for use in HA or HI tests for at least the next 3 weeks without further washing or standardization. It is stored at 2–4°C.

CHEMICAL REAGENTS

All reagents are prepared in glass-distilled or demineralized water.

Borate-saline, pH 9.0. This solution is 0.05 M borate-0.12 M NaCl. It is prepared from concentrated stocks by diluting 80 ml of 1.5 M NaCl, 100 ml of 0.5 M H_3BO_3 and 24 ml of 1.0 M NaOH to one L with water.

Borate-saline, pH 9.3. This is prepared as above except that the volume of 1.0 M NaOH is 34 ml for 1 L.

Acid-citrate-dextrose, ACD. Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)—11.26 g, citric acid ($H_3C_6H_5O_7 \cdot H_2O$)—4.0 g, dextrose—11.0 g. This is made to 500 ml with water and sterilized by autoclaving for 10 minutes at 10 pounds pressure.

Dextrose-gelatin-veronal, DGV. The veronal (barbital) used in this reagent is crystallized once by chilling a saturated solution of commercial barbital (USP) made in hot ethyl alcohol. The sodium veronal is prepared from USP grade barbital by making a saturated solution of the latter in ethyl alcohol at *room temperature* and precipitating the sodium salt by the gradual addition, with stirring, of an alcoholic solution of NaOH (1.5–2.0 M) until the suspension is alkaline (blue) to Universal indicator. The crystals are, in each case, collected on a Buchner funnel and dried.

One liter of DGV solution contains the following components: veronal (barbital) 0.58 g, gelatin 0.60 g, sodium veronal (sodium barbital) 0.38 g, $CaCl_2$ (anhydrous) 0.02 g, $MgSO_4 \cdot 7H_2O$ 0.12 g, NaCl 8.5 g, dextrose 10.0 g.

The veronal and gelatin are dissolved in 250 ml of water with the aid of heat. This solution is combined with the other reagents, dissolved in water in a 1-L volumetric flask, and the whole is made to volume with water. The reagent is sterilized by autoclaving at 10 pounds pressure for 10 minutes, following which the pH should be 7.0 to 7.6.

Antigen-serum diluent, bovalbumin borate saline

TABLE 2

Adjusting diluents for addition of cell suspensions

Final pH	% 0.15 M NaCl-0.2 M Na_2HPO_4	% 0.15 M NaCl-0.2 M NaH_2PO_4
5.75	3.0	97.0
6.0	12.5	87.5
6.2	22.0	78.0
6.4	32.0	68.0
6.6	45.0	55.0
6.8	55.0	45.0
7.0	64.0	36.0
7.2	72.0	28.0
7.4	79.0	21.0

The pH is that obtained by mixing equal volumes of borate-saline, pH 9.0, and adjusting diluent.

pH 9.0. For tests in lucite plates, the presence of a certain amount of added protein is necessary to obtain optimal settling patterns and to prevent nonspecific cell agglutination. Even in tubes, the tests are frequently improved by the added protein. There is some suggestive evidence that bovine albumin also aids in decreasing the temperature dependence and increasing the pH range of the hemagglutination reaction. For plate tests, a 0.4 per cent solution of bovine albumin (Armour fraction V) in borate saline is used. A 0.2 per cent solution is satisfactory for tests in tubes. To avoid a decrease in the pH of the borate saline due to the acid albumin, a 4 per cent stock of bovine albumin is made in borate saline and the pH adjusted to 9.0 with 2 M NaOH. The working solution is prepared from the stock by dilution in borate saline pH 9.0.

Adjusting diluents for addition of cell suspensions. These are 0.15 M NaCl-0.2 M phosphate and are most easily prepared from two reagents, one of which is 0.15 M NaCl-0.2 M Na_2HPO_4, and the other, 0.15 M NaCl-0.2 M NaH_2PO_4. We find it convenient to prepare these two solutions, in turn, from accurately-made concentrated stocks of NaCl, Na_2HPO_4 and $NaH_2PO_4 \cdot H_2O$. Table 2 gives the formulae for adjusting diluents to cover the pH range 5.75 to 7.4. It must be stressed that the pH values given are those resulting from the mixture of equal volumes of borate saline pH 9.0 and the adjusting diluents.

TECHNIQUE OF HA AND HI TESTS

All arbor-virus antigens are quite stable in borate saline at pH 9. Held in this solution, even at high dilution, they show no change in titer over a period of hours at room temperature or overnight at 2 to 4°. Antigen-serum mixtures can therefore be incubated in a refrigerator overnight, with considerable enhancement of antibody titer. The phenomenon of hemagglutination, however, is markedly dependent on pH, does not occur with most viruses much above pH 7, and is usually maximal below that level. In the absence of receptor erythrocytes, many of the viral agglutinins are rather unstable under these conditions. For this reason, the final pH adjustment of the antigen or antigen-serum mixture is made simultaneously with the addition of the erythrocytes. This is accomplished by adding the cells suspended in a buffered diluent which is sufficiently acid to yield the exact pH required (adjusting diluent—see section on chemical reagents). The optimal pH is dependent not only upon the virus under study but also on the method of antigen preparation, the presence or absence of bovine albumin in the diluent, and the temperature of incubation. It is, however, highly reproducible for different lots of the same virus prepared and tested in the same manner. For most viruses, the optimal pH falls in the range 6.0 to 7.0.

The temperature of incubation of the test, after the addition of erythrocytes, is either 37°C or room temperature. Low temperatures are avoided because they retard cell settling rates and the resulting patterns are frequently poor. As a general rule we incubate most tests in lucite plates at 37°C (air incubator), although room temperature is satisfactory for many viruses. Tests in tubes are incubated at room temperature, except that for those antigens which yield significantly higher titers at 37°C a water bath at that temperature is used.

The choice of plates versus tubes appears, at the present time, to be a matter of individual preference. For large-scale work, plates offer great advantages over tubes in the convenience of handling and cleaning. The plates are obtained from Prestware, Ltd., South Down Works, Kingston Road, Raynes Park, London, S.W.20, and are described as "agglutination trays $5\frac{11}{16}$" x $7\frac{1}{16}$" containing 80 spherical cavities $\frac{3}{4}$" in diameter." These are the plates designed originally for influenza virus work. The tubes are 13 x 75 mm, round-bottom hemagglutination tubes obtained from Scientific Glass Apparatus

Company, 100 Lakewood Terrace, Bloomfield, N. J.; specify: as made for The Rockefeller Institute. Both plates and tubes are best decontaminated and cleaned by soaking in Clorox which inactivates the virus and dissolves the deposit of red cells. Tubes are emptied of their fluid contents and immersed in 10 per cent Clorox in a horizontal position for a few hours, after which they are rinsed with tap water and cleaned in the familiar dichromate-sulfuric acid cleaning solution used for any type of glassware. Plates are stacked on edge in 10 per cent Clorox and held overnight, following which they are brushed vigorously with soap and water using a large scrubbing brush. They are then washed thoroughly with tap and distilled water.

HA TITRATIONS

HA titrations are regularly done on all new lots of antigen prior to their use in HI tests. With viruses not previously studied, such titrations are done over the entire known pH range of activity and at both room temperature and 37°C. With viruses for which the optimal conditions are known, it is sufficient to check the titer at one temperature and at a few pH values at, and around, the previously determined optimum. Most hemagglutinating viruses titer out to quite high levels (giving positive reactions when diluted several hundred or even thousand-fold). A few new viruses have yielded quite low-titer antigens which have, nevertheless, been of great value in virus classification. It is wise, therefore, to begin the titration of new antigens at a 1:10 dilution.

The antigen is diluted 1:10 (or higher for known high-titer antigens) in chilled, bovine-albumin-borate-saline, pH 9, and the diluted antigen is held in an ice bath. Serial 2-fold dilutions are made in the same diluent at room temperature. If a new virus is under study, titrations are carried out at the following pH values: 5.75, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0 and 7.4. It is necessary, then, to have 16 identical sets of dilutions in the pH 9 diluent, eight to cover the pH range at room temperature and eight at 37°C. In addition, one plate well, or tube, containing only pH 9 diluent for each pH and temperature, serves as a cell control to assure that proper settling patterns are being obtained under all test conditions. The antigen dilutions are best made in master tubes and subsequently distributed in 0.4-ml amounts into the plate wells. The same procedure may be used for tubes except that 0.5-ml volumes are delivered, or else serial dilutions can be made directly in the HA tubes by use of an automatic pipetting device. In our experience, the most satisfactory apparatus for serial dilution is a 2-ml B-D Cornwall continuous pipetting outfit[4] from which the filling tip containing the valves has been removed. The plunger is lubricated with a very small amount of mineral oil and vaseline, and a short piece of thick-walled, narrow-bore, rubber tubing is placed over the Luer-Lok syringe tip. The rubber tubing should project a few millimeters beyond the syringe tip so that uncalibrated Pasteur pipettes can be inserted easily into the tubing and held securely. The apparatus is adjusted to deliver a volume of 0.5 ml and will be found to make quite accurate serial dilutions at that volume.

After the completion of the antigen dilutions in plates or tubes, goose erythrocytes are added in the various adjusting diluents to give the required pH values. It will be recalled that the cells are stored in suspension at a known concentration as determined by optical density (diluted 1:40, the optical density is 0.450). For tube tests, the desired optical density is 0.450, while for plates it is 0.750. The stock cells are therefore diluted 1:40 in the various adjusting diluents for tube tests or 1:24 for tests in plates. Since the adjusting diluents are both acid and hypertonic, it is important to chill these solutions and to minimize the time elapsing between the preparation of the final suspensions and their addition to the tests; otherwise, the erythrocytes may be damaged. Cell suspensions in adjusting diluents that give final pH values from 5.75 to 6.6 can be held in ice baths, but it is advisable to avoid extreme chilling of the higher pH adjusting diluents since crystallization of Na_2HPO_4 may occur. The cell suspensions, in volumes of 0.4 ml for plates or 0.5 ml for tubes, are best added by means of a B-D Cornwall continuous pipetting outfit (1-ml or 2-ml size), used as designed by the manufacturer. Use of this device is essential for tests in plates in order to obtain adequate mixing, and greatly facilitates the work if any considerable number of tubes is used.

After addition of the cells to all antigen dilutions and cell controls, the plates are placed at

[4] Becton, Dickinson and Company, Rutherford, New Jersey.

the desired temperature and read after a period of 1 to 2 hours. The racks of tubes are shaken briskly prior to incubation. Tests in plates are read from above, the plates being placed over a white background. Visualization of tube tests is simplified if the racks are placed above a slanted mirror held in a frame so that the bottoms of the tubes can be viewed by reflection. In control and negative tubes, the cells settle as dark red, compact, central buttons. Complete agglutination is manifest by a uniform, thin, translucent shield of cells covering the entire lower surface of the well or tube. An incomplete shield combined with a dark ring of cells indicates partial hemagglutination. Any desired system for scoring the results may be used; the one used most extensively in these laboratories is: + = complete agglutination; ⊕ = partial agglutination; ± = trace agglutination; 0 = no agglutination. In all tests, it must be shown that the cell controls are completely negative.

HI TESTS

It is generally much more convenient to prepare the sera by kaolin treatment and erythrocyte adsorption a day or so ahead of time. On the day of the test, one or more ampules of each antigen are rehydrated with cold water and held in an ice bath. On the basis of previous titrations, a dilution of each antigen is made in cold bovine-albumin-borate-saline to give an estimated 4 to 8 units of hemagglutinin per 0.2 ml or 0.25 ml volume, depending on whether plates or tubes are used. A sufficient volume is prepared to allow the addition of 0.2 ml or 0.25 ml to all serum dilutions and also to permit preliminary and final antigen titrations to be carried out. It is our practice to allow the diluted antigens to stand for about 1 hour in an ice bath prior to titration in order to permit complete dissociation of any aggregates which may exist in the concentrated stock. A preliminary titration of each diluted antigen is then made at the pH and temperature previously found to be optimal. If the observed titer is either too high or too low, adjustment is made by the addition of more diluent or of concentrated antigen.

The test sera are diluted serially in 2-fold steps in the pH 9.0 diluent. This is done in master tubes at room temperature, the usual dilution range being 1:10 to 1:5,120. The serum dilutions are then transferred to the appropriate plate wells or tubes (0.2-ml volumes for plates, 0.25-ml for tubes). In addition, serum controls are prepared by combining at least one 0.2-ml or 0.25-ml sample of each serum at 1:10 (or the lowest dilution employed) with an equal volume of the pH 9 diluent in a plate well or tube. The serum controls serve to assure that nonspecific agglutinins are absent from all test specimens. One control for each serum is probably adequate if the final test conditions for all antigens are fairly similar as to pH and temperature. If, however, different temperatures and rather widely divergent pH values are to be used, more than one such control should be included. After all serum dilutions have been distributed, 0.2-ml or 0.25-ml volumes of each antigen, containing 4 to 8 units of hemagglutinin, are added to the plates or tubes. In addition, a 0.4-ml or 0.5-ml sample of each antigen is added to an equal volume of pH 9.0 diluent in a well or tube. The resulting preparations are held overnight with the rest of the test and serve to initiate the final antigen titrations which are carried out on the second day, prior to the addition of the erythrocytes. The antigens are added to plate tests by means of a 1-ml size Cornwall continuous pipettor. They may be added to tubes in the same manner, or a graduated pipette may be used.

After all antigens have been added, the test is incubated overnight in an ordinary refrigerator at 2 to 4°C. In order to minimize evaporation during the overnight incubation and also during the incubation following the addition of the cells, plates are stacked one on another with a blank plate on top. On the second day, the test is taken out of the refrigerator, dilutions for the final antigen titrations are carried out, and 0.4 ml or 0.5 ml of the appropriate cell suspensions are added to give the required pH and optical density as described for HA titrations. After incubation at room temperature or 37°C, readings are made as for HA titrations. Inhibition of hemagglutination is indicated by a negative reaction (button of cells) and the HI titer is taken as the highest dilution of each serum which causes complete or almost complete inhibition of 4 to 8 units of antigen. For a test to be considered entirely satisfactory, all cell and serum controls must be negative and each final antigen titration should show the presence of 4 to 8 antigen units. If a final antigen titration is somewhat too high or too low, the observed serum titers may be corrected,

since a regular reciprocal relationship exists between antigen units and HI titer. If, however, less than two full agglutinating units are present, the serum results should be considered suspect, particularly those which show low HI titers.

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BOOKS RECEIVED

The Relation of Psychiatry to Pharmacology, by Abraham Wikler, M.D. 322 pages. Baltimore, Williams & Wilkins Company, 1957.

Diagnostic Medical Parasitology, by Edward K. Markell, Ph.D., M.D. 276 pages, ill. Philadelphia, W. B. Saunders Company, 1958, $7.00.

BOOK REVIEWS

Introduction to Statistical Analysis, 2nd edition, by WILFRID J. DIXON, Professor of Biostatistics, and FRANK J. MASSEY, Associate Professor of Biostatistics; University of California, Los Angeles. New York, Toronto and London: McGraw-Hill, 1957. Pp. xiii, 488. $6.00.

The limited mathematical preparation of the student and the need for some understanding of the theoretical justification of methods of analysis are the two sides of a dilemma that one has to face in writing a textbook in applied statistics. The student in an applied field is not often accustomed to mathematical manipulations, whereas a pure description of statistical methods may very well turn the book into a collection of recipes. The authors of the second edition of *Introduction to Statistical Analysis* handled this dilemma quite beautifully. Although a student with a meager "knowledge of algebraic addition, subtraction, and multiplication" may not necessarily find all parts of the text easy to follow, most of the arguments are presented with a minimum amount of mathematics but with adequate rationale. The fine treatments of analysis of variance and the Neyman-Pearson theory of testing hypotheses are good examples. Since the book is intended to be a textbook for students in applied fields, derivation of formulas and tests is kept to a minimum.

The 20 chapters of the book cover a wide range of topics. In addition to descriptive statistics, notion of population and sample, normal distribution, statistical inference, analysis of variance, regression and correlation, the chi-square test, and the binomial and Poisson distributions, it contains a lucid account of the often-neglected analysis of covariance, all-important power function, increasingly-popular nonparametric statistics, and ever-useful sequential analysis. The chapter on nonparametric statistics covers the sign test, run test, rank-sum test, median test, and other commonly used nonparametric tests. The material is well organized and well presented. It is indeed a good chapter on the subject for a textbook at this level.

The main features of the second edition of the book are the revision of the chapters on statistical inference and on analysis of variance, the addition of a chapter on probability, a list of 133 references, and the extension of the tables to a whopping 113 pages. It is safe to say that the list of tables is the most extensive that one can find in a textbook in statistics at the present time, and both the teacher and student will find it extremely useful.

The chapter on analysis of variance is completely rewritten and several new topics are added. The inclusion of the section on individual comparisons is especially noteworthy for, in the analysis of experimental data, the investigator is often interested in the significance of the difference between specific pairs of means in addition to the test of homogeneity of means of all the groups under study. Without knowledge of the tests described in this section, he frequently appeals to the so-called "least-significant-difference" (L.S.D.), a notion based upon unsound statistical reasoning.

A significant change in this chapter is made regarding the use of the F test in two-way classifications with more than one observation in each cell. In the first edition of the book, it was recommended that the interaction be tested first, and that the statistic used to test the row (or column) means be determined by the outcome of the test for interaction. If the interaction is significant, the rule given is to use the interaction mean square in the denominator of the F-ratio to test row (or column) means; otherwise, the residual (interaction + within-groups) mean square is to be used. In the second edition, however, the use of the within-groups mean square alone in the denominator is suggested. This is regardless of the interaction. The case when interaction is significant is not discussed. There are theoretical justifications for the use of within-groups mean square for testing the hypothesis of homogeneity of row (or column) means regardless of the significance of the interaction. For in Model I of the analysis of variance, the expectation of the row (or column) mean square is independent of the interaction variance; also, if the interaction is not zero, the interaction mean square (upon division by σ^2) does not have the chi-square distribution, and thus the

ratio of row (or column) mean square to the interaction mean square will not have the F distribution. On the other hand, there are practical arguments in favor of using the interaction mean square and these were given in a convincing manner in the first edition. A loyal student, who has been following the procedure in the first edition of the book, will be bewildered to find a different approach in the second edition. It would seem helpful to discuss the logic of both approaches so that the student will see the difference and understand the logic on which the preference is based.

The chapter on probability is the new chapter in the second edition of the book. In view of the fact that probability is an extremely important and almost indispensable concept in the understanding of statistics, the new chapter is most welcome. In a first course in applied statistics, some students may find probability problems a stumbling block, but there are other students who find this subject interesting and even fascinating, and consider it a necessary change in taste after tedious calculations of mean, standard deviation, and the like. In this new chapter there are introduced not only the concept of probability but also that of probability density function, distribution function, and expected value of a random variable. Thus, the addition of this chapter makes the book quite complete as a textbook in elementary statistics.

There are always points in a book on which the reviewer disagrees with the author. The following are some of these points by way of commenting on the chapter on probability. (1) This chapter properly begins with the introduction of the concept of equally likely events and of relative frequency. But nowhere in this 29-page chapter is there a definition of the term probability. (2) Although an elegant derivation of the Poisson formula as a limiting case of the binomial distribution is given in the latter part of this chapter, the inequalities $0 \leq P(A) \leq 1$ are considered as an axiom "in order not to overcomplicate the presentation." It is unnecessary to point out that more mathematical training is needed to understand the derivation of the Poisson formula than is needed to follow the argument that the values of a relative frequency are bounded by zero and one. (3) There are no multiplication theorems for the case of two mutually independent events or for the case of more than two events. (4) The terms "complementary," "exhausive," "independent," and "exclusive events" are introduced in the same sentence and followed immediately with definitions of the latter three; complementary events are not defined until the closing paragraph of the next section. (5) There is a considerable amount of repetition here of material first presented in Chapter 13 on the binomial and the Poisson distributions, but no mention is made of the earlier presentation. (6) The expectation of random variable is introduced in the last section of the chapter. A more logical place perhaps after discussion of random variable and frequency function (Sec. 20-4). As it stands, no examples of expectations of random variables are given, although both the binomial and Poisson distributions are only a few pages away. This perhaps is the first textbook in statistics ending with the introduction of the concept of expectation. These points need not be interpreted as a criticism of the presentation in this chapter but rather they represent a difference between the authors and the reviewer in their approach to the subject.

The first edition of the book had been very well received. With the significant changes made in the second edition, the book is even more suitable for the student and the research worker in the applied field.

CHIN LONG CHIANG

Studies on the Exo-Erythrocytic Cycle in the Genus Plasmodium, (London School of Hygiene and Tropical Medicine Memoir 12), by R. S. BRAY, B.Sc., Ph.D., 1st edition, 192 pages, illustrated. London, H. K. Lewis & Co., Ltd., 1957, £2.2s. net.

This volume is a valiant effort to provide an up-to-date account of a subject not previously adequately summarized. The work, in the author's words, "cannot be treated other than as an interim report on a subject swiftly growing after thirty years in the wilderness (1903–1933)." Following two short introductory chapters on "Definitions, general classification and terminology" and "Introductory notes on the plasmodia" there is, in Chapter three, an excellent historical summary of the subject, covering the early period of knowledge of exo-erythrocytic (e-e) schizogony of the genus *Plasmodium* (1890–1934) and the later history of the e-e cycle in avian plasmodia (1934–1943) and mammalian plasmodia (1934–1947). The remaining four-fifths of the volume is a detailed account of the present state of knowledge of the e-e cycle of avian and saurian plasmodia (Chapter 4) and mammalian plasmodia (Chapter 5), with a short final chapter on "Taxonomy and specific classification." This latter portion of the work is extremely ambitious in its scope and in many ways is stimulating to the reader.

In Chapter four the author gives a detailed account of the e-e cycles of *Plasmodium gallinaceum* and includes briefer descriptions of other

avian and saurian species, relating their characteristics to those of *P. gallinaceum*. Similarly, in Chapter five there is a detailed description of the e-e cycle of *P. cynomolgi*, this forming a basis for comments concerning the other mammalian species. In these same chapters are included discussions of the relationship of the tissue-dwelling forms of the parasite to relapses, immunity, pathogenicity, chemotherapy, etc. Although this work is most commendable in its aims and completeness, there are a few faults which might be pointed out.

At the outset it is unfortunate that the author's effort to allow "last-minute research to take its place in the text" is hampered by a 3- to 4-year delay in publication of the volume. Only a small amount of work published as late as 1954 is included. While the author promises "new information which might otherwise never reach the interested reader," the reviewer could find only two original experiments of relatively minor importance described. The author's more recent and excellent work in malaria in chimpanzees would have added considerably to the volume and to the fund of knowledge in this field.

It is also unfortunate that the author indulges so frequently in the expression of personal opinion. Indeed his bent for disagreement seems at times quixotic. Because of these tendencies it is most difficult for one to review the work with complete objectivity. While the reviewer would not disagree with the author's right or ability to express these opinions, it is felt that such is not in keeping with the stated aims of the work or in the interest of an objective review of the field.

The author's reasoning is frequently suspect and again often seems to be colored by opinion. On page 134, as the only support of a contention that the tissue phase of *P. falciparum* ceases after the first generation he states that no such tissue stage has been found "in thousands of liver autopsies performed *post-mortem*" on persons dead of this disease. In succeeding paragraphs the continuous and active e-e schizogony of *P. vivax*, *P. malariae*, and *P. ovale* is described, but without an explanation as to the total absence of autopsy findings in persons dead of these infections. The possibility of an initial pre-erythrocytic cycle during the first 48 hours after sporozoite inoculation of mammalian plasmodia appears to be ruled out by the author principally on the basis of failure to detect forms of any kind during this period. The use in this work of the term "exo-erythrocytic stages" interchangeably either to mean all stages other than erythrocytic or only those stages which occur subsequent to the pre-erythrocytic cycle is frequently confusing. Since the author is not averse to proposal of changes in other areas, a proposal for elimination of this confusing terminology might have been welcome.

The work is well documented and has an extensive bibliography of 628 titles. While the reviewer checked only a few references, it appears that there are a large number of errors in the Bibliography and references thereto. The author's frequent use of "personal communication" and "private communication" is regrettable, especially since it is known that the sources of these communications were sometimes conversational and not documented. The figures are in some cases excellent, but a few are not expertly done (e.g., Fig. 21) and some show few of the stipulated details (Figs. 2, 5, and 16).

The usual high quality of printing and binding which characterizes the Memoir series is found in this volume.

For all of its faults this work will serve a very useful purpose in bringing together much of the information on the exo-erythrocytic cycles in *Plasmodium* published through 1953. It will also serve as a valuable index of the areas where further investigations are needed to fill gaps in our knowledge of these cycles. The book is commended to all persons engaged in research in malaria, with the hope that it will be as stimulating to them as it was to this reviewer.

G. M. JEFFERY

My Arabian Days and Nights, by ELEANOR T. CALVERLY, M.D., 182 pages. New York, Thomas Crowell Company, 1958, $2.95.

The experiences of the first woman physician in primitive, poverty-stricken Kuwait from 1912 to 1929 are high-lighted by contrasts seen on a recent visit to this now oil-rich country. A personal account of the struggle to introduce Western medicine to Moslem women reveals many interesting glimpses of their family life, although most of the customs described will be familiar to those who have read at all widely in this field. Little of technical value in Tropical Medicine is included beyond mention of the various diseases encountered.

The author, whose husband opened a Mission school for boys, had to practice independently and in a separate part of the building from that used by the other Mission doctor who treated the men. From a description of preparing in trepidation for her first major operation with only uneducated assistants, the story progresses with many an aside about raising a family, social visits with Arab and British officials, and warfare among desert tribes, to an account of practice in a hospital assisted by a trained nurse. The final account of a return visit in 1955 reveals the great enlightenment which had occurred in the inter-

vening years along with glimpses of certain aspects of life which had shown little change.

J. ALLEN SCOTT

The Bacteriology of Tuberculosis, by EGONS DARZINS, M.D. 488 pages, illustrated. Minneapolis, University of Minnesota Press, 1958, $10.00.

This book offers the reader a wealth of information concerning the tubercle bacillus and experimental tuberculosis. The subject matter is organized in a logical fashion. It begins with a description of the tubercle bacillus and its growth requirements. It reviews methods of isolation and identification of tubercle bacilli and describes their effects on experimental animals. The material is presented in historical perspective and is well documented.

Those interested in basic bacteriology will find a comprehensive description of the biological, chemical and physical factors associated with identification of cell structures. Those interested in diagnostic methods will find a thorough review of the microscopic and cultural techniques for isolation and identification of the tubercle bacillus. Those interested in experimenting with the tubercle bacillus will find reference to the fundamental techniques currently employed in studying tuberculosis in animals. The reader will find the section on hazards and precautions in the laboratory of great practical value.

It is obvious that Dr. Darzins has had an extensive experience in studying the bacteriology of tuberculosis. His book will prove to be most useful to physicians, scientists and technicians who still seek further advances in the treatment and eventual elimination of tuberculosis.

MARTIN M. CUMMINGS

The Medical and Veterinary Importance of Cockroaches, by LOUIS M. ROTH AND EDWIN R. WILLIS. 147 pages, illustrated. Washington, D. C., Smithsonian Institution, 1957.

The authors have reviewed the literature dealing with the transmission of various pathogenic organisms and parasites by cockroaches. There is an impressive list of such organisms that have been associated with these insects; the associations have been established by experimental infections and by recovery of the organisms from wild cockroaches. The authors conclude, "There is no question about the ability of cockroaches to carry pathogens in or on their bodies", but that there is ". . . some question about the epidemiological significance of the fact." As is true of flies, the filthy habits of the pest cockroaches, especially their inhabiting of sewerage systems, make them potential carriers of pathogens from feces to food destined for human consumption.

The organisms associated with cockroaches include viruses, bacteria, fungi, protozoa, and helminths. The review includes sections on allergy, cockroach bites, accidental invasion of man, cockroaches as human food, and cockroaches in medicine and folklore. In 5 appendices, there are presented lists of organisms, vertebrate hosts, and cockroach associations. To this reviewer, this is the most useful part of the book, especially Appendix E, which presents a compilation of the literature on the helminths transmitted by cockroaches. Unlike the other organisms, which usually are carried by cockroaches mechanically under special circumstances, there are a number of helminth parasites of animals which utilize these insects as intermediate hosts.

L. E. ROZEBOOM

The Epidemiology and Control of Malaria, by George Macdonald, C.M.G., M.D., F.R.C.P., Director of the Ross Institute of Tropical Hygiene; Professor of Tropical Hygiene, London School of Hygiene and Tropical Medicine. 201 + liv pp., illustrated with figures and tables. London, New York and Toronto, Oxford University Press, 1957. Price, $7.50 in U.S.A.

From the time of Hippocrates to the etiological discoveries of the last quarter of the 19th century the epidemiology of malaria, without knowledge of parasite or vector, concerned itself perforce with the circumstances under which the disease occurred and spread. When the discoveries of Laveran and Ross disclosed the full cycle of transmission, the old "circumstantial" epidemiology gave way to the studies of the parasite and the vector which constitute the great advance of the last half century. Nevertheless, the mass of detailed knowledge accumulated by this "biological" approach to epidemiology has not provided a satisfactory understanding of the diverse manifestations of the disease under the almost infinite number of possible combinations of biological and circumstantial variables. Professor Macdonald now attempts to identify and evaluate the measurable factors influencing the transmission and endemicity of malaria, and thus to provide a scientific basis for the explanation of the differences in the malaria picture under varying ecologic conditions. Such a numerical approach introduces a sense of proportion and an order of importance into the jumble of observed phenomena, and tends to clarify the underlying causes. It may be called "mathematical" epidemiology, whose aim is to integrate biological and circumstantial data into a coherent science of epidemiology providing a rational understanding of the disease.

The word "mathematical" need not alarm the individual who finds biostatistical analysis hard to follow. The author has succeeded extraordinarily well in giving a lucid verbal account of the quantitative aspects of transmission, endemic equilibrium, and the nature of epidemic malaria which anyone can comprehend, and has reserved for an appendix the more abstruse mathematical statements needed for a complete understanding of the argument as a basis for critical examination and further development.

The quantitative aspects of the epidemiology of malaria have stemmed until recently from the original concepts of Ross whose mathematical treatment of the factors in transmission, though unassailable, was based on the fragmentary knowledge of 50 years ago. The present extension of the method to cover most of the known variables is novel and stimulating. One need not accept all the suggested applications of theory to practice to realize that a new perspective is offered of great practical utility. No clearer description of the complicated epidemiology of malaria or the relatively simple underlying principles has, in my opinion, ever been made. Their application to measures for malaria control or eradication will prove extremely valuable. An appendix gives details of the techniques necessary for the epidemiological study of parasites and mosquitoes, and of the newer ones which the use of insecticides has made indispensable. No one concerned with the epidemiology of malaria or with the strategy of its control can afford to disregard the contributions of this book.

L. W. Hackett, M.D.

NOTICE TO CONTRIBUTORS

The Journal welcomes papers which contribute to the purpose of the American Society of Tropical Medicine and Hygiene. This is the advancement of knowledge of tropical and subtropical medicine and hygiene through investigation, prevention, treatment and control of diseases and deficiencies. It draws upon medicine, biology, public health, nursing, engineering and allied fields of research and application.

The American Journal of Tropical Medicine and Hygiene combines the American Journal of Tropical Medicine and the Journal of the National Malaria Society and therefore has a special concern with the investigation, therapy and control of malaria in all parts of the world, as well as a broad interest in general parasitology and arthropod-borne diseases.

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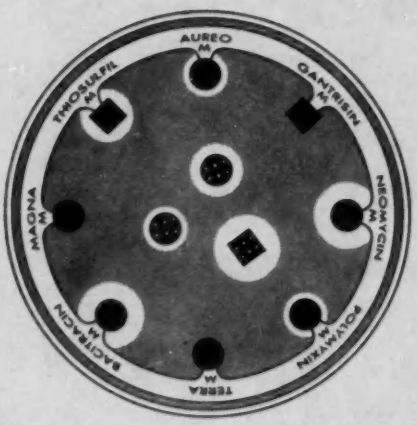

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